Analysis of the Plasma-Cell Repertoire in Inflammatory Myopathies

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Abbreviations

AID	activation-induced cytidine deaminase
ADCC	antibody-dependent cell-mediated cytotoxicity
APRIL	a proliferation-inducing ligand
BAFF	B-cell-activating factor
BCR	B-cell receptor
CD	clusters of differentiation
CDR	complementarity determining region
CFU	colony forming units
CSF	cerebrospinal fluid
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
D	diversity
DC	dendritic cell
DNA	deoxyribonucleicacid
DM	dermatomyositis
EBV	Epstein-Barr virus
eGFP	enhanced green fluorescence protein
ER	endoplasmatic reticulum
FACS	fluorescence-activated cell sorting
FDC	follicular dendritic cell
FR	framework
GC	germinal center
hc	heavy chain
HCMV	human cytomegalovirus
IBM	inclusion-body myositis
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
IM	inflammatory myopathies
IRES	internal ribosome entry site
J	joining
κc	kappa chain
λc	lambda chain

lc	light chain
LTR	long terminal repeat
mDC	myeloid dendritic cell
MG	myasthenia gravis
MOG	myelin oligodendrocyte glycoprotein
MOI	multiplicity of infection
MS	multiple sclerosis
PC	plasma cell
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PF	perifascicular
PM	polymyositis
PNA	peanut agglutinin
R	replacement mutation
RA	rheumatoid arthritis
RNA	ribonucleicacid
RT	reverse transcriptase
S	silent mutation
SHM	somatic hypermutation
SLE	systemic lupus erythematosus
TNF	tumor-necrosis factor

Summary

Inflammatory myopathies (IM) can be divided into three distinguishable syndromes, dermatomyositis (DM), inclusion-body myositis (IBM), and polymyositis (PM). Current knowledge suggests DM to be a primarily antibody-mediated autoimmune disease. While the immunopathogenesis of IBM and PM appears to be mediated by cytotoxic $CD8^+$ T-cells, IBM additionally shows signs of degenerative processes. However, recent data have demonstrated the presence of B cells and plasma cells in diseased muscle derived from DM, PM, and IBM patients. The major goal of this work was to characterize the repertoire of immunoglobulin (Ig) genes expressed by B- and plasma cells in IM to gain further insight in B-cell-dependent mechanisms possibly playing a role in the etiology of IM. In order to achieve this goal, we established a method permitting the isolation of individual plasma cells from muscle biopsy material followed by single-cell RT-PCR of Ig heavy and light chain genes.

From forty-one IM biopsy specimens we identified eleven biopsy tissues, including samples from DM, IBM, and PM patients by means of immunohistochemistry, which contained $CD19^+$ B cells and $CD138^+$ plasma cells. Biopsies containing B- and plasma-cells were first analyzed by CDR3 spectratyping to obtain an overview of the clonal composition of the Ig repertoire contained in the biopsy material. Subsequently performed single-cell RT-PCR of Ig genes from laser-microdissected plasma cells revealed a strong clonal expansion of plasma-cell clones in IBM, whereas in DM and PM we found a more polyclonal Ig gene repertoire. While in IBM Ig heavy chain sequences derived from the same plasma-cell clone were mostly identical, some sequences derived from DM and PM showed signs of clonal variation. Some of the strongly expanded plasma-cell clones we identified in IBM tissue by single-cell RT-PCR, we could confirm by the methodologically independent CDR3 spectratyping analysis. Our data provide insight into the Ig repertoire of infiltrating plasma cells in IM and indicate an antigen-driven immune response involving B cells in affected muscle, especially in IBM. Furthermore, the amplification of both Ig heavy and light chain genes from the same cell permits the recombinant resurrection of the original antibody specificity, providing a powerful tool to identify potential target antigens of muscle-directed, autoaggressive immune responses. Such tools should greatly enhance our understanding of IM immunopathogenesis, possibly opening the route for the development of novel therapeutic approaches.

Zusammenfassung

In der Krankheitsgruppe der inflammatorischen Myopathien unterscheidet man drei Formen: Dermatomyositis (DM), Einschlusskörpermyositis (inclusion-body myositis, IBM) und Polymyositis (PM). DM wird als eine primär Antikörper-vermittelte Autoimmunerkrankung angesehen, während bei IBM und PM zytotoxische $CD8^+$ T Zellen für die Immunpathogenese verantwortlich zu sein scheinen. Bei der IBM sind zusätzlich degenerative Veränderungen nachgewiesen worden. Neue Erkenntnisse deuten darauf hin, dass B-Zellen und Plasmazellen, die vor kurzem im Immunzellinfiltrat bei allen inflammatorischen Myopathien nachgewiesen wurden, eine mögliche Funktion in der Pathogenese haben.

Das Ziel der vorliegenden Arbeit war die Charakterisierung des B- und Plasmazell-Repertoires anhand der Analyse ihrer Immunglobulingene, und ihrer möglichen Funktion in der Ätiologie der IM. Dazu wurde eine Methodik etabliert, die die Isolierung von einzelnen Plasmazellen aus Muskelbiopsien und die Amplifikation der schweren und leichten Immunglobulin (Ig) Kettengene möglich macht. Aus einer grösseren Anzahl von Biopsien wurden elf Gewebe ausgewählt, in denen B- und Plasmazellen immunhistochemisch nachgewiesen werden konnten. Zunächst wurden B-Zell positive Biopsien mit Hilfe des CDR3-Spektratype Verfahrens analysiert, um eine Gesamtübersicht über die klonale Zusammensetzung des darin enthaltenden Immunglobulinrepertoires zu erhalten. Anschliessend wurden an ausgewählten Biopsien einzelne Plasmazellen mit Hilfer der Laser-Mikrodissektion isoliert und per Einzelzell-RT-PCR analysiert. Die Resultate der Einzelzell-RT-PCR der Immunglobulingene ergaben starke Expansionen von Plasmazellklonen in IBM-Geweben und ein eher polyklonales Immunglobulinrepertoire in DM und PM. Während die Analyse der schweren Immunglobulinkettengene der stark expandierten Plasmazellklone in IBM-Gewebe fast ausschliesslich identische Sequenzen aufwiesen, zeigten einige aus DM and PM Gewebe amplifizierte Sequenzen die Zeichen von klonaler Variation. Einige der stark expandierten Plasmazellklone im IBM Gewebe konnten mit der methodisch unabhängigen CDR3-Spektratype Methode bestätigt werden.

Unsere Daten geben Aufschluss über das Immunglobulinrepertoire der Muskel-infiltrierenden Plasmazellen und deuten auf eine Antigen-induzierte humorale Immunantwort insbesondere bei der IBM hin. Darüberhinaus erlaubt die Amplifikation der schweren und leichten Ig Kettengene aus derselben Zelle die Wiederherstellung der ursprünglichen Antigenspezifität des von dieser Plasmazelle sezernierten Antikörper durch rekombinante Expression. Diese Technik kann die Identifizierung von neuen Zielantigenen bei Myositiden ermöglichen, unser

Wissen über die Immunpathogenese der IM vergrössern und möglicherweise Wege für die Entwicklung neuer therapeutischer Ansätze eröffnen.

1 Introduction

1.1 The humoral immune system

1.1.1 Biology of B cells

The adaptive immune system, containing T-lymphocytes, B-lymphocytes and their secreted antibodies as key elements, plays an important role in eliminating foreign microorganisms and molecules that may otherwise compromise health and wellbeing. B lymphocytes arise from hematopoietic stem cells and, after a clonal selection process, mature to produce antibodies specific to an antigen. In the bone marrow, stem cells mature independent of an antigen into pro-B cells, pre-B cells, and immature B cells, which enter the antigen-dependent phase of development in the peripheral lymphoid tissues (Figure 1).

1.1.2 Antibodies

Antibodies are the effector molecules of B cells which are located on the cell-surface or can be secreted. They are able to bind surface antigens of pathogens, such as fungi, bacteria, and viruses and contain two different functional parts, the antigen-binding fragment (Fab), and the constant fragment (Fc), which interacts either with other immune cells or activates the complement cascade to opsonize and finally eliminate the pathogen. Antibody molecules are heterodimers consisting of two heavy-chain and two light-chain molecules which are joined by disulfide bonds. There are antibodies of IgM isotype, which can also form pentamers. Each chain has a variable region and a constant region (Han et al., 1995). The variable region is responsible for the binding of antigen. The heavy chain (hc) is comprised by three constant regions CH1-CH3 and one variable region; the light chain (lc) by one variable and one constant region. The variable region of light chains consists of a combination of alleles from only two families (V) and (J) which can originate from two different genetic loci (kappa or lambda). The hypervariable region of hcs is generated by recombination of three different genetic elements: The variable (V), diversity (D), and joining (J) gene segment (Early et al., 1980).

The diversity of the immunoglobulin repertoire is established by four processes. First, by the recombination of three or two gene segments, which constitute the V-region of heavy and light chains, respectively. In these recombination events, different gene segments are randomly combined (combinatorial diversity). Second, junctional diversity is generated during recombination by addition or depletion of nucleotides at the joints between different gene segments. Further diversity is generated by the combination of different heavy- and

light-chain variable regions. Combinatorial and junctional diversity leads to the generation of approximately 10^{11} different receptors. Finally, somatic hypermutation (SHM) introduces mutations into the variable region genes during affinity maturation and increases further the diversity of the antibody molecules. The constant region is responsible for the effector functions of the antibody. This part of the antibody is coded by mainly five different C region genes and determines the isotype.

1.1.3 Antigen-independent development in the bone marrow

The development of lymphocytes in mammals takes place in highly specialized environments of the central lymphoid organs. B cells and T cells originate from hematopoetic stem cells in the bone marrow. Later T cells migrate into the thymus. Both immune cell subset development is characterized by gene rearrangement of their receptors. The state of development of both cell types can be determined by their rearrangement status. After reaching adolescence, T cells no longer develop in the thymus, but in peripheral lymphoid organs. B cells are produced lifelong in the bone marrow. In both specialized lymphoid compartments non-lymphoid cells, so-called stromal cells are present which are essential for the development of the B and T cells and interact with these cells through the secretion of growth factors and expression of signaling molecules on their cell surface (van Ewijk, 1991; Funk et al., 1994).

Some of these bone marrow-derived factors and their functions are known. For example the growth of early B-lineage cells is induced by stem cell factor (SCF), a membrane-bound cytokine present on stromal cells. This factor interacts with the cell-surface receptor tyrosine kinase (Kit) on B-cell precursors. Later in development other factors are required, like the cytokine interleukin-7 (IL-7) or the chemokine stromal-cell derived factor-1 (SDF-1) (Nagasawa et al., 1996).

The antigen specificity of each individual T cell and B cell is determined by the rearrangement of V-, D-, and J-gene segments. The expression of the whole receptor molecule of a B cell requires the successful rearrangement of two gene loci, one for the heavy chain and one for the light chain immunoglobulin gene. A successful rearrangement (productive rearrangement) leads to the entry of the cell into the next stage of development. An „out-of-frame rearrangement", which does not lead to protein expression is called a non-productive rearrangement and leads to apoptosis. The individual stages in primary B-cell development are defined by the sequential rearrangement and expression of heavy and light chain immunoglobulin genes (Janeway CA et al., 2001).

The first classification is made by the expression of either heavy chain alone (pre-B cell) or heavy and light chain (immature B-cell) expression.

The earliest B-lineage cells are called pro-B cells. They are derived from pluripotent hematopoietic stem cells and are identified by expression of characteristic cell surface markers e.g. MHC class II, CD10, and CD19. In this stage, the D_H to J_H rearrangement in the early phase takes place. At a later stage the V_H to D_HJ_H joining takes place. Productive rearrangement leads to the expression of a functional heavy chain, which characterizes pre-B cells. These cells express the heavy chain in combination with a surrogate light chain. Pre-B cells start to rearrange the light-chain locus and, after successful joining, a complete IgM molecule is expressed. The cells are now called immature B cells (Hardy et al., 1991).

1.1.4 Antigen-dependent development in the periphery

The entire development up to this stage has taken place within the bone marrow, independent of antigen. Now immature B cells are selected for self-tolerance and the ability to survive in peripheral lymphoid tissues. Only 50% of the pre-B cell population emerges from primary development in the bone marrow. It is assumed that this is due to the failure in making a productive light-chain rearrangement. Most peripheral B cells are long-lasting and only 1-2% of these cells die every day. The immature B cells, which circulate outside the bone marrow, are further tested for self-tolerance and look for survival niches within B-cell follicles in secondary lymphoid organs, like the lymph nodes. In these follicles, follicular dendritic cells (FDCs) and T-helper cells provide B cells with survival signals. If immature B cells are unable to get into these follicles, 50% of the population dies within 3 days. Because of the low probability that a helper T-cell will find the appropriate B cell, it was long a major question as to how this might happen. It is assumed that when an animal is immunized with an antigen, the antigen is captured, processed, and presented in context with MHC class II molecules on professional antigen presenting cells, such as dendritic cells. These cells migrate until antigen encounter into the T-cell zones of lymph nodes. Naive circulating T cells migrate through such lymph nodes and encounter dendritic cells which present their antigen. A mechanism similar to that of T-cell trapping is assumed for the trapping of B cells. On encountering their antigen, B cells are arrested by the activation of adhesion molecules and the engagement of chemokine receptors (such as CCR7). In this manner antigen-binding B cells are precisely trapped in the correct location to maximize the probability of encountering a helper T-cell which activates them. The interaction with helper T cells activates the B cell to establish a primary focus of clonal expansion.

When these positively selected B cells are re-stimulated with the relevant antigen, clonal expansion takes place. This leads to mature (naive) B cells expressing surface IgM and IgD, later developing into activated B cells, in the germinal center into memory B cells or early and late plasmablasts. Plasmablasts finally mature into antibody-producing PCs that are all specific for the original antigen (Figure 1) (Goldsby R.A. et al., 2000; Avery et al., 2005; Sell and Max, 2001). Specific cluster of differentiation (CD) markers such as CD19 and CD20 distinguish the B cells from stem cells and PCs (CD138).

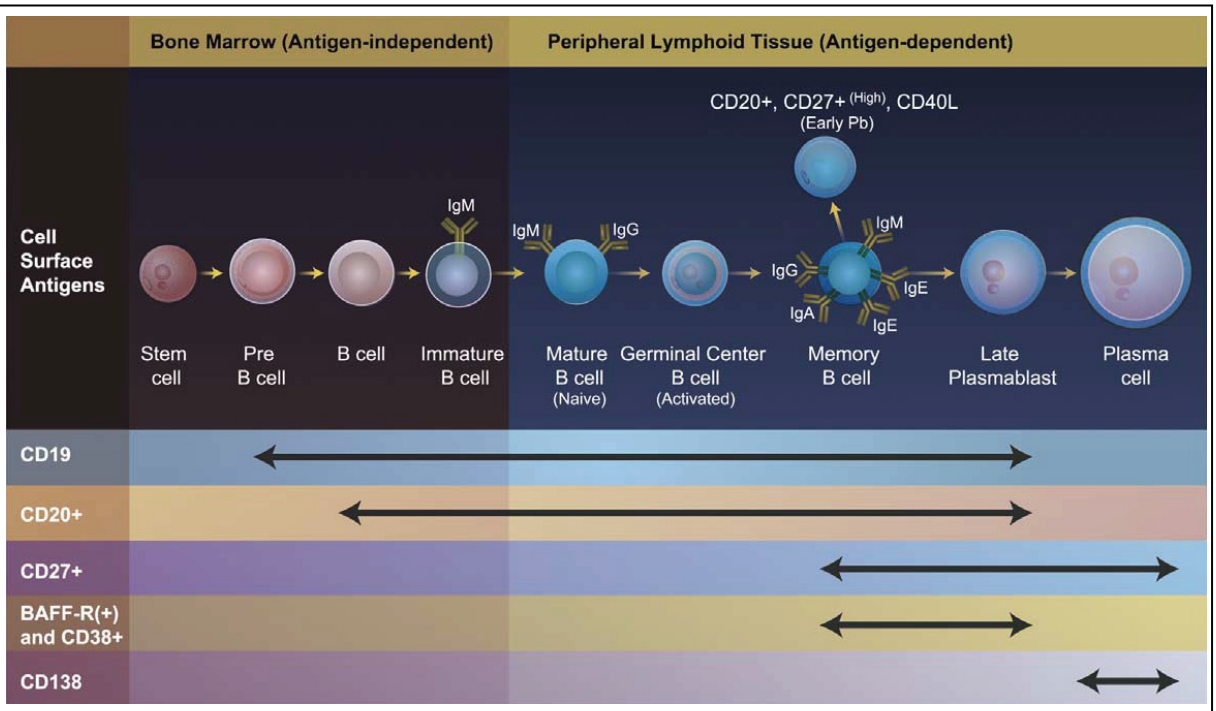


Figure 1. Development of B cells (adapted from Dalakas et al. 2006).

1.1.5 Germinal center reaction

Some B cells and T cells which have proliferated within the primary focus migrate into a primary lymphoid follicle, where they continue to proliferate and form a germinal center (GC), which serves as a cellular environment for the improvement of affinity through SHM and class switch recombination of immunoglobulin genes. A GC is a complex cellular aggregate of many different cell types. This specialized microenvironment can be divided into a dark zone with rapidly dividing centrocytes and a light zone in which small centrocytes are in contact with a network of FDC processes. Antigen-specific helper T-cells which are in strong contact with proliferating GC B cells as well as follicular dendritic cells (FDCs) which present antigen on their surface are important for the GC response (Manser, 2004).

T-helper cells in the GC express CXC-chemokine ligand 13 (CXCL13) (Kim et al., 2004) at high levels to recruit B cells to the GC and the adaptor protein SAP (signaling lymphocyte-activation molecule (SLAM)-associated protein), which is required for providing help (Crotty et al., 2003).

Interactions between the B-cell receptor, CD40 ligand and CD40 and inducible T-cell co-stimulator (ICOS)-ICOS ligand which are present at the cell surface of helper T- and B cells take place to maintain the GC reaction (Bishop and Hostager, 2001). The signals from the B-cell receptor and CD40 are assumed to upregulate the expression of Bcl-X_L which prevents apoptosis of B cells. FDCs sequester antigen in the GC for which complement receptors (CD21 and CD35) expressed by FDCs are crucial. (Barrington et al., 2002). It is assumed that such sites of antigen persistence are survival signals for long-lived PCs.

Antigen-specific B cells that participate in the GC reaction expand rapidly and downregulate their germline encoded BCR (MacLennan, 1994). The clonal expansion is accompanied by BCR diversification through SHM (Jacob et al., 1991; Berek et al., 1991). Interestingly, SHM has also been demonstrated outside GC (Matsumoto et al., 1996; William et al., 2002). SHM is characterized by the introduction of single base-pair substitutions with rare insertions and deletions into the variable regions of antibody gene segments. The main enzyme involved in this process is the recently discovered activation-induced cytidine deaminase (AID) (Revy et al., 2000). B cells which have improved their affinity receive further survival signals, whereas cells with lower affinity to their antigen undergo apoptosis.

1.1.6 Ectopic germinal center reaction

Affinity maturation of B cells and the final development into either a memory B cell or a PC usually takes place in secondary lymphoid organs, for example in lymph nodes or in the spleen. In several autoimmune and also infectious diseases however, structures are present in the affected organs that resemble GCs, for example, in diarthrodial joints in rheumatoid arthritis (Takemura et al., 2001), the thyroid gland in Hashimoto´s thyroiditis (Armengol et al., 2001), the thymus in myasthenia gravis (Roxanis et al., 2002), the salivary glands in Sjogren´s syndrome (Amft et al., 2001), and the meninges in multiple sclerosis (Prineas, 1979; Serafini et al., 2004) as examples for autoimmune diseases. They are also found in chronic inflammatory diseases, e.g. in the gut in Crohn´s disease (Carlsen et al., 2002), and also in infectious diseases in the stomach in Helicobacter pylori induced gastritis (Mazzucchelli et al., 1999). These structures are called ectopic GCs or tertiary lymphoid organs. They are characterized by the presence of B cells, PCs, T cells, FDCs, and high endothelial venules, as well as by the expression of the chemokines CXCL12 and CXCL13. See as an overview of an ectopic GC development figure 2. For more information see review article (Aloisi and Pujol-Borrell, 2006).

The genetic analysis of V-genes from B cells, isolated from GC-like structures, has shown SHM. It is assumed that these B cells mature at such ectopic sites into PCs and produce high-affinity autoantibodies which might bind to antigens in the affected organ.

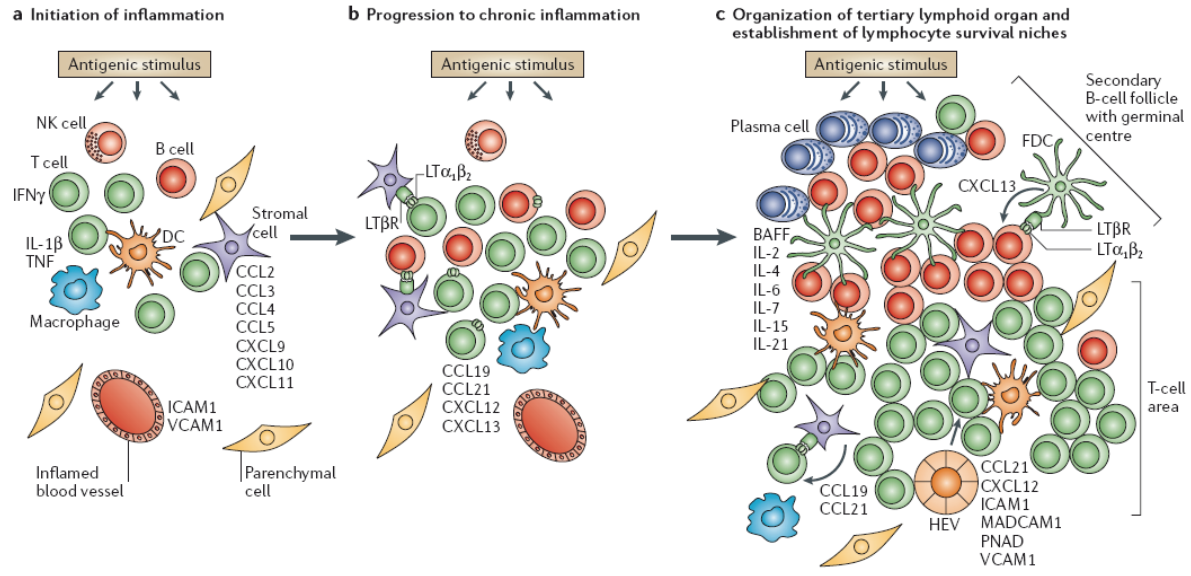


Figure 2. Schematic model of cellular and cytokine–chemokine networks in the initiation of inflammation and in the development of chronic inflammation and lymphoid neogenesis (adapted from Aloisi et al. 2006).

1.2 B cells and autoimmunity

1.2.1 Plasma cells

After several days of proliferation of both B cells and T cells, the primary focus begins to disappear and many lymphocytes comprising the focus undergo apoptosis. However a small fraction of dividing B cells will differentiate into antibody-synthesizing PCs and migrate to the red pulp of the spleen or the medullary cords of the lymph nodes. PCs can further develop from naive marginal-zone B cells, from follicular B cells, from activated GC B cells, and from memory B cells (Shapiro-Shelef and Calame, 2005).

The differentiation into PCs is mediated by the activation of Blimp-1, a transcriptional repressor (Turner, Jr. et al., 1994) downregulating the expression of two transcription factors that are required for GC reactions, BCL-6 (Shaffer et al., 2002) and PAX5 (Lin et al., 2002), thereby ensuring that, once PC development has been initiated, B cells cannot return to an earlier developmental stage. PCs are highly Ig-secreting cells, with abundant cytoplasm containing multiple layers of rough endoplasmic reticulum. Ig makes up 10-20% of synthesized protein. The surface expression of Ig is still present but very low. It seems necessary for the PC to bind antigen on the surface to receive survival signals. PCs lack surface expression of MHC class II molecules but are still sensitive to signals like IL-6 and CD40L. PCs have different life spans, which may vary between days and several years (Janeway CA et al., 2001).

1.2.2 Plasma cells and autoantibodies

Recent literature provides evidence for the involvement of B cells in the immunopathogenesis of autoimmune disorders, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and myasthenia gravis (MG) (Drachman, 1994; Lipsky, 2001; Edwards et al., 1999). Under physiological conditions the human immune system produces by random recombination of V-, D-, J-gene segments antibodies which are autoreactive. This phenomenon is suspected to help in maintaining the optimal functioning of the immune system. There are tolerance-inducing mechanisms at key points of B-cell development. If these regulatory mechanisms are compromised, loss of self-tolerance may be of disease initiating consequence.

In the past, the role of B cells in autoimmunity has been associated mainly with the ability of PCs and plasmablasts to produce self-damaging antibodies. These autoantibodies may bind to self-antigens and may interfere normal cellular functions and/or lead to tissue destruction e.g. by activating the complement cascade. Examples are, the deficit of acetylcholine

receptors in MG or the deposition of autoantibodies against basement membrane collagen type IV in the kidney and lung alveoli in patients with Goodpasture`s syndrome or anti-glomerular basement membrane (GBM) syndrome, induces glomerulonephritis and necrotizing hemorrhagic interstitial pneumonitis by complement activation and recruitment of immune cells (Dalakas, 2006a). In dermatomyositis (DM) it is assumed that antibody-induced complement activation leads to destruction of muscle capillaries, thus inducing the characteristic perifascicular atrophy seen in DM biopsies (Dalakas and Hohlfeld, 2003).

Furthermore, B cells participate in immune reactions not only by autoantibody production, but also by activating immune cells through secretion of proinflammatory cytokines, antigen presentation and complement activation. For example, B cells activate the secretion of IL-6, TNF-α, and IL-10 by macrophages and modulate the function of other immune cells (Fillatreau et al., 2002; Duddy et al., 2004; Chan et al., 1999; Weinstein et al., 2004; Goldsby R.A. et al., 2000).

Through antigen presentation to naive T cells, B cells can stimulate their antibody production, by direct interaction as well as by cytokine production (Constant, 1999). This interaction can lead to the expansion of B cells and T cells which recognize the same epitope in parallel. This phenomenon has been shown for the recognition of myelin basic protein (MBP) epitopes in MS patients (Wucherpfennig et al., 1997). The Fc portion of an antibody can also bind to its receptor on macrophages, neutrophils, and NK cells. These activated cells attack the specific tissue by antibody-dependent cell-mediated cytotoxicity (ADCC).

BAFF, a potent B-cell supporting factor, plays a role in such diseases by activating the expression of oncogenes like Bcl-2 (Mackay and Tangye, 2004). Bcl-2 expression leads to the survival of B cells. In the context of autoimmune-diseases it can be hypothesized that uncontrolled BAFF expression can lead to the survival of autoreactive B lymphocytes. Increased BAFF expression has been observed in the CNS of MS patients (Krumbholz et al., 2005).

Depletion of B cells in autoimmune diseases by an anti-CD20 antibody rituximab (MabThera®; Rituxan®), is beneficial in RA and is currently being tested in other autoimmune disorders including multiple sclerosis. This treatment does result in a decrease of autoantibody concentration (RF), whereas the total Ig serum levels decrease only slightly. Additionally, levels of pneumococcal-polysaccharide-specific antibodies are not changed during treatment, because these antibodies are derived from marginal-zone B cells which seem not to be targeted by rituximab (Edwards et al., 2004).

1.2.3 Plasma-cell migration and survival niches

PCs migrate from secondary lymphoid organs to the bone marrow via blood or the lymphatic system. There is ever-growing understanding of the mechanisms which direct cells to homage sites. Adhesion molecules, such as VLA-4, VLA-5, CD9, CD44, and CD138 (Arce et al., 2004; Ridley et al., 1993; Wehrli et al., 2001), are present on the PC surface and are assumed to be involved in their homing and survival in specific tissues.

Also, chemokine receptors play a role in regulating the migration and survival of PCs which are attracted by the expression of CXCL12, the ligand for CXCR4 in the red pulp of the spleen, medullary cords of lymph nodes, and the bone marrow stroma (Hargreaves et al., 2001). While on plasmablasts CXCL12 has a migration-stimulating effect, PCs respond by prolonged survival (Hauser et al., 2002).

The signals which are present in the bone marrow for long-term survival are not completely understood. Some factors, however, have been identified which prolong survival, such as IL-5, IL-6, CXCL12, SDF-1α, and TNF-α (Roldan and Brieva, 1991; Wols HAM et al., 2002; Cassese et al., 2003).

CXCR3 is another chemokine receptor which is expressed by PCs (Hauser et al., 2002; Manz et al., 2002). The corresponding ligands (CXCL9-CXCL11) are expressed in inflamed tissues and by some high endothelial venule cells, which are present in the draining lymph nodes of B-cell follicles (Janatpour et al., 2001). PCs are present in chronically inflamed tissues (Cassese et al., 2003; Mallison, III et al., 1988).

1.3 Inflammatory myopathies

The inflammatory myopathies (IM) can be divided into three distinguishable syndromes: polymyositis (PM), dermatomyositis (DM), and inclusion-body myositis (IBM) (Dalakas and Hohlfeld, 2003; Dalakas, 2004).

DM leads to proximal muscle weakness and skin changes, affecting both children and adults. PM is a rare disease affecting mostly adults and leads to proximal muscle weakness, as well. IBM mostly affects adults and is characterized by proximal and distal muscle weakness. Its progression is slow. All three disorders are characterized by an inflammation of the endomysium, muscle-fiber necrosis, and fibrosis (Dalakas, 1998; Dalakas, 2006c).

1.3.1 Dermatomyositis

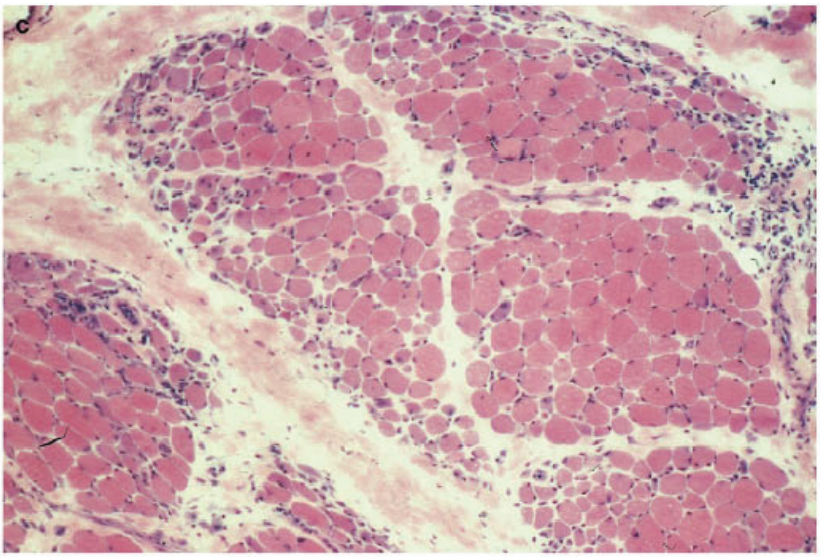


Figure 3. Perfascicular myofiber atrophy in dermatomyositis muscle (adapted from Dalakas et Hohlfeld 2003).

In DM, the intramuscular capillaries are characterized by endothelial hyperplasia with tubureticular profiles, vacuolization, and necrosis, which is thought to cause ischemia and the characteristic muscle-fiber atrophy at the periphery of muscle fascicles (perifascicular myofiber atrophy see figure 3) (Kissel et al., 1986). The serum level of the muscle specific creatine kinase, which is released from necrotic muscle fibers is increased, and correlates to disease activity. Complement deposition and activation of C5b-9 membrane attack complex on endomysial blood vessel walls and additionally of immune complexes consisting of Ig and complement C3 are part of the first-occurring immunopathological changes which lead to destruction of capillaries.

B cells are a frequently found cell type within the cellular infiltrate in DM, which is also characterized by high CD4/CD8 ratio and B cell and CD4 T-cell synapses, which would suggest a CD4 T cell-induced Ig secretion by B cells (Hohlfeld and Engel, 1994). One general hypothesis in the pathogenesis of DM is tissue damage by way of autoantibody-mediated complement activation (Dalakas and Hohlfeld, 2003; Whitaker and Engel, 1972; Kissel et al., 1986).

Additionally, perifascicular fibers express MHC class I antigens and various IFN-γ or α and β induced markers, such as CD56 and amyloid beta precursor protein (ABPP). The inflammatory cellular infiltrate also contains plasmacytoid dendritic cells (pDCs) (Greenberg et al., 2005b). These histological findings and data from microarray experiments indicate a type 1 IFN-driven immune response and also suggest an innate immune response in contrast to the long-established antibody-mediated autoimmune paradigm in DM.

1.3.2 Inclusion-body myositis and polymyositis

In PM and IBM cytotoxic $CD8^+$ T-cells are thought to be responsible for muscle fiber destruction (Dalakas and Hohlfeld, 2003). These cells are localized around and inside MHC class I antigen-expressing non-necrotic muscle fibers. In both diseases the muscle fibers are thought presenting antigen and form immunological synapses with the T-cell receptors of clonally expanded $CD8^+$ T-cells (Hofbauer et al., 2003). In PM as well as in DM, it was demonstrated that perforin is expressed in muscle infiltrating T cells (Goebels et al., 1996). However, only in PM a vectorial orientation of intracellular perforin containing granula onto myofibers have been demonstrated, suggesting perforin to be an important effector molecule in the pathogenesis of PM. The infiltrating and long-persisting $CD8^+$ T cells are in an activated state because they express CD45RO, ICAM, ICOS, and MHC class I antigen. The upregulation of MHC class I antigen expression on the target cell is typically found in PM and IBM and induced by cytokines. In noninflammatory chronic myopathies the class I antigens are absent (Michaelis et al., 1993; Wiendl et al., 2005). The MHC class I expression is present on every muscle fiber in IBM and PM, regardless of whether they are surrounded, invaded, or free of inflammation. This observation suggests two processes in parallel: 1. a cytotoxic T-cell response triggered by MHC class I presentation and the presence of costimulatory molecules. 2. a non-immune process which is characterized by ER stress induced by overexpression of MHC class I antigen (Dalakas, 2006c).

In PM and IBM, the data suggests a HLA-restricted, antigen-specific, T-cell-mediated myocytotoxicity. Therefore, the $CD8^+$ T cells presumably recognize antigens, which are expressed and processed in the endogenous pathway of antigen presentation, by the

antigen-presenting cell itself. It is further assumed that these proteins may be viral components or from self (Hohlfeld and Engel, 1994). Until now, there is no evidence for the presence of viral antigens or genomes in muscle fibers (Leff et al., 1992; Dalakas, 2006b). This would argue for a T cell-mediated autoimmune response in PM and IBM.

In IBM, diagnostic markers are the presence of filamentous inclusions (which contain myxovirus nucleocapsids), and of 15-21nm cytoplasmic tubulofilamentous inclusions which consist of ubiquitin and the accumulation of 6-10nm β-amyloid-like filaments (Massa et al., 1991; Askanas et al., 1991). The presence of ABPP, microtubule-associated protein tau and ubiquitin clearly argues for a degenerative component in the pathogenesis of IBM.

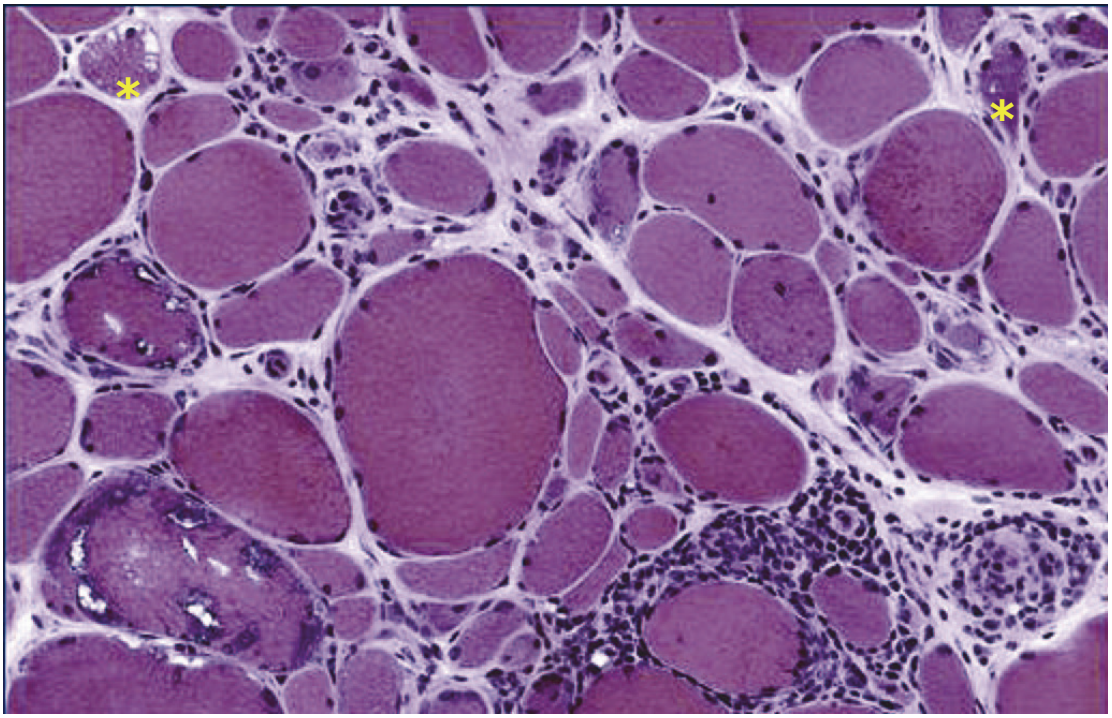


Figure 4. Cross-section of a muscle from a patient with IBM
Note two red-rimmed vacuolated fibers (yellow asterix) not invaded by inflammatory cells. These vacuolated fibers contain degeneration-associated molecules, such as ABPP, ubiquitin, or tau. Adapted from (Dalakas, 2006b).

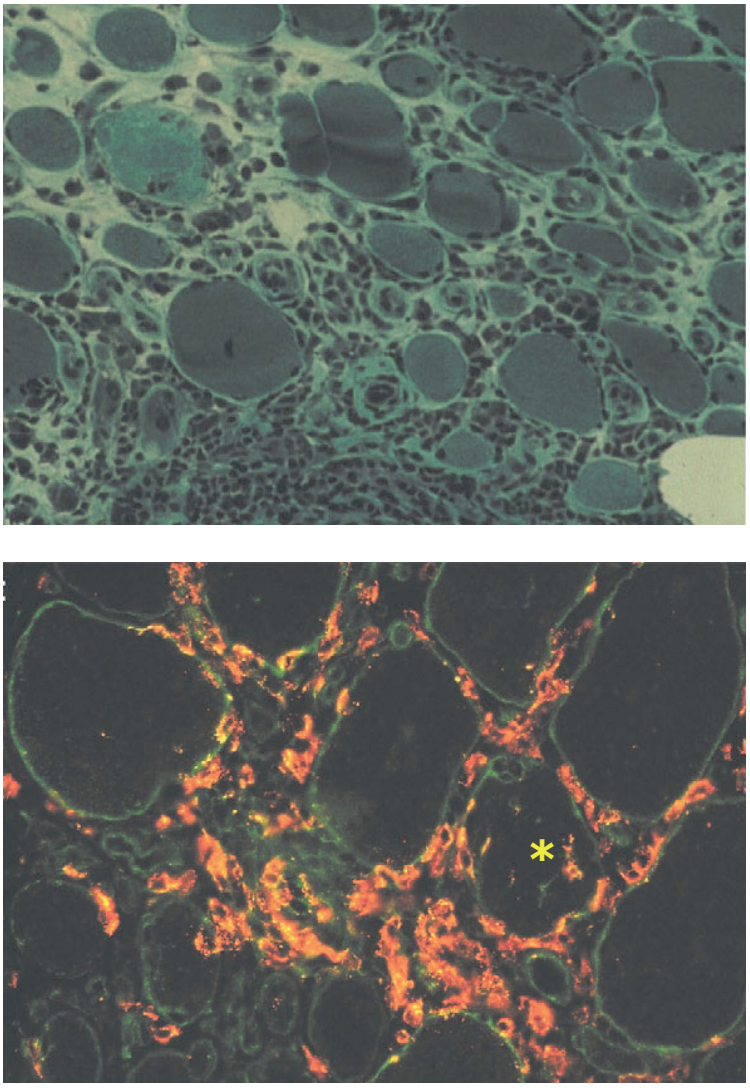


Figure 5. Infiltration of immune cells in IBM and PM.
Above: Endomysial inflammation in polymyositis and inclusion-body myositis with lymphocytic cells invading myofibers (yellow asterix).
Below: The MHC-I/CD8 complex in polymyositis and inclusion-body myositis. MHC-I (green) is upregulated on muscle fibers, and CD8-positive T cells (orange), that also express MHC-I, invade the fibers. (Adapted from (Dalakas and Hohlfeld, 2003)).

1.3.3 Inflammatory myopathies and humoral immunity

All three diseases are assumed to be (auto-) immune mediated diseases in which the target antigens have not been identified. Greenberg et al. have recently shown that the immune cell infiltrate in inflammatory myopathies may also contain plasma cells (Greenberg et al., 2005a). Recently, the infiltrating PC repertoire in DM and IBM was analyzed by generating cDNA libraries of heavy chain Ig genes from inflammatory myopathy tissue. The analysis of the immunoglobulin variable region sequences indicates an antigen-driven immune response, indicated by clonal expansion of B cells and affinity maturation, which is characterized by the insertion of replacement mutations within the Ig sequences. Analyses of B cells from different tissue regions show clonal variation, which the authors discuss as a clue of affinity maturation within the muscle tissue itself and outside from classical sides of GC reaction (Bradshaw et al., 2007).

1.4 Identification of antigens by expression cloning

In our lab we generate recombinant human antibodies derived from expanded plasma-cell clones from several presumed autoimmune diseases like multiple sclerosis, myasthenia gravis and inflammatory myopathies. The final goal of these studies is the identification of the target antigens and the analysis of their pathophysiological relevance. Therefore, we sought to generate a system in which possible target antigens are expressed in a structure as similar to the human system as possible.

The cellular processes comprise an enormous number of specific protein-protein and protein-DNA interactions for the cellular functions of e.g. protein expression, cell-cycle and cell-cell interaction, and connected signaling pathways. One major goal in cell biology is the knowledge of all molecular interactions of a cell. This would lead to the development of new compounds for the treatment of diseases. To discover interacting partners and ligands several approaches over the last two decades have been developed.

The identification of binding partners for distinct target molecules is one of the main goals in molecular biology. It is also relevant for the development of drugs, in which molecules have to be identified for their impact on cellular interactions. For this reason, different methods have been developed to achieve these goals. The more simpler systems use cDNA libraries generated from a target tissue or cell type and cloned into prokaryotic vectors for the expression in bacteria. Other systems utilize lambda phages for the generation of fusionproteins with phage core proteins. The advantages of such systems are that they are easy-to-handle and well-established. However, proteins expressed in prokaryotic libraries have a number of disadvantages such as the absence of posttranslational modifications and proper folding of proteins. More advanced mammalian expression cloning systems using viral mediated gene transfer have been established recently (Koller et al., 2001).

The main advantages are the introduction of the transgene into the genome and the resulting stable expression of the protein. Furthermore, viral infection allows the adjustment of the infection rate per cell to approximately one insertion of the retroviral construct into the genome per cell. That allows the identification of antigens more easily compared to transfections in which high copy numbers of plasmids can enter one cell and protein expression will be lost after a short period of time. We therefore planned to generate an expression system, in which cDNA libraries of human origin are retrovirally transferred and expressed in mammalian cells.

1.5 Goal of this work

The initial aim of this thesis was to characterize the B- and plasma-cell infiltrate in dermatomyositis, the one IM which had traditionally been assumed to be antibody and complement-mediated. The recent finding of infiltrating PCs in all three IM disorders encouraged us to also include PM and IBM in our studies. Using two different methodological approaches, we aimed to characterize the clonal composition of the B and plasma-cell infiltrate in IM muscle specimens. The two methods we chose are complementary: CDR3 spectratyping is a molecular tool which gives an overview on the total clonal composition contained in the tissue volume analyzed, based on the CDR3 length distribution of expressed V_H chain genes. On the contrary, the single-cell RT-PCR analysis can only be performed on a limited number of individual plasma cells excised by laser microdissection from tissue sections. Yet it allows to obtain both immunoglobulin heavy and light chain gene sequences from individual plasma cells, which is the basis to reconstruct the antigen specificity of their secreted immunoglobulins by means of expression as recombinant monoclonal antibodies. These antibodies could then be used to screen expression libraries derived from IM mRNA for their cognate target antigen(s) and to identify the possible role of the humoral immune system in the pathogenesis of IM.

2 Materials & Methods

2.1 Biopsy collection

Muscle specimens obtained from biopsies were provided by the Friedrich-Baur-Institute, University Hospital Munich. Muscle biopsies were performed for clinical indications, independent of the presented study, which was approved by the local ethics committee.

2.2 Immunohistochemistry

The immunohistochemistry was performed by the „Labor für Spezialtechniken" at the Department of Pathology (University Hospital Zurich). Following antibodies were used: CD3, CD4, CD8, CD10, CD20, CD38, CD68, CD138, C3C, anti-IgG, anti-IgM, and anti-IgA.

Antigen	Cell specificity	Clone	Dilution	Detection system	Source
CD 3	T cells	SP7	1:100	Ultra View DAB	Labvision
CD 4	T cells	1F6	1:30	Ultra View	Medite
CD 8	T cells	C8/114B	1:100	Ultra View	Dako
CD 20	B cells	L26	1:100	Dako En Vision	Dako
CD 68	macrophage	PG-M1	1:50	Ultra View	Dako
CD 138	Plasma cells	MI15	1:30	Ultra View	Dako
IgA	B cells	polyclonal	1:20.000	iView DAB	Dako
IgG	B cells	polyclonal	1:15.000	iView DAB	Dako
IgM	B cells	polyclonal	1:5.000	iView DAB	Dako
(PNA)	GC B cells	na	1:400	Vectastain Elite HRP	Vector

Table 1. Antibodies used for immunohistochemistry.

2.3 Immunofluorescene stainings

a) Double immunofluorescence staining of CD138 / HLA-DR

Myositis tissue (snap frozen) was cut into 10µm thick sections using a cryotome (Leica) at -25°C. After a two-step fixation in acetone 50% v/v in water for 30s followed by 100% acetone for 5 minutes at 4°C, sections were washed in TBS pH 7.6 and incubated with normal goat serum (5% v/v in antibody diluent, Dako Cytomation) for 30 minutes at room temperature (RT). Endogenous Biotin was blocked by applying Streptavidin Solution (Avidin/Biotin blocking kit, Vector Laboratories) for 15 minutes and after rinsing incubated

with Biotin Solution for 15 minutes. After two steps of washing with TBS, sections were incubated with biotinylated mouse anti-CD138 (clone B-B4, Diaclone) antibody diluted 1:25 in antibody diluent (Dako) and anti-HLA-DR FITC (Dako) antibody diluted 1:25 for 2 hours at RT. After two steps of washing (2x 10 minutes in TBS), sections were further incubated with a Streptavidin-594 (Alexa Fluor 11227, Molecular Probes) conjugate diluted 1:700 in antibody diluent. The sections were washed twice (10 min) in TBS, incubated with Hoechst dye to stain the nucleus, then finally washed and embedded in Medi-Mount (Medite).

b) Staining of plasma cells for laser microdissection application

Snap-frozen tissue specimens were cut into 10μm- to 12μm-thick sections, using a cryotome (Leica) at -25°C. Tissue pieces were placed onto metal frame slides (Molecular Machines), then thawed for approximately 10s at room temperature. The staining procedure was done according to the protocol of the HistoGene LCM immunofluorescence staining Kit (Arcturus). Tissues were fixed in acetone (Merck) on ice for 2 min. The slide was kept at room temperature until the acetone had evaporated completely, then put onto a metal plate cooled on ice. The tissue section was circumscribed using a PapPen (Dako) and rehydrated with staining buffer. Biotinylated anti-CD138 antibody (clone B-B4, Diaclone) was diluted 1:4 in staining buffer and after adding Prime RNase inhibitor (Eppendorf) to an final concentration of 2U/μl applied to the tissue for 4 min. After three washing steps (adding each time 150μl of ice-cold staining buffer), the section was incubated with Cy3-Streptavidin 1μl diluted in 200μl of staining buffer (Arcturus Kit) for one minute. After three more washing steps as described above, the section was dehydrated for 30 seconds each in 70%, 95%, and 100% ethanol. After 5 min clearing in xylene, the section was dried for 10 min and used directly for laser microdissection.

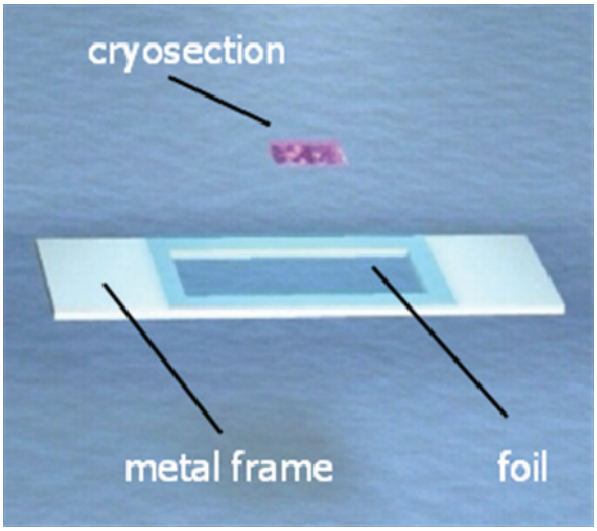


Figure 6. Overview of slide preparation for LM.

2.4 CDR3 spectratyping

This PCR-based method gives insight into the clonal composition of a lymphocyte population. It is based on the natural length polymorphism of the CDR3 region of hc Ig genes. As an output of this method the distribution of the different V_H genes is given according to the nucleotide length of their CDR3 region. In the case of a polyclonal V_H repertoire, the V_H sequence-length representation would lead to a Gaussian distribution (Figure 7), if the y-axis represents the relative concentration and the x-axis the nucleotide length of the PCR products in the amplicon, whereas a clonally expanded V_H repertoire would be represented as single peaks on the plot. The clonal composition can be analysed at the V_H and J_H family levels. Spectratyping permits the detection of clonal distribution with a resolution of each V_H and J_H Ig families. For more information on CDR3 spectratyping see (Pannetier et al., 1995).

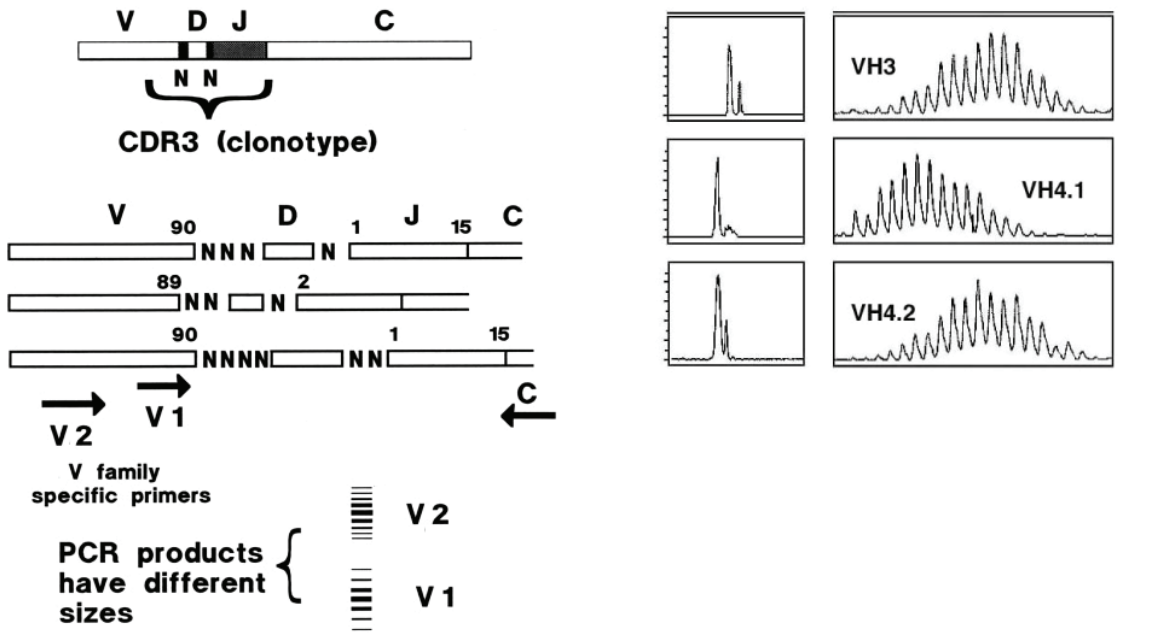


Figure 7. Principle of CDR3 spectratyping.
Left: Schematic drawing of the CDR3 region: Random insertion of (non genomically encoded) N-nucleotides between the genomically encoded V-, D-, and J-regions during the process of somatic recombination leads to a natural length polymorphism of the CDR3 region of the rearranged T-cell receptor beta chain and immunoglobulin heavy chain gene. (modified according to Gorski et al., 1995).
Right: Example of CDR3 spectratyping patterns. Whereas the three panels on the right show the typical Gaussian distribution of polyclonal B-cell populations, the three left panels show examples of strongly expanded B-cell clones (shown here is the analysis of CSF cells from an MS patient).

For CDR3 spectratyping approximately 15 sections (each of 10μm thickness) were cut using a cryotome, and lysed in Trizol (Invitrogen) for the isolation of total RNA. Trizol containing lysed tissue, was incubated for 5 min at RT, chloroform was added and vigorously shaken. After 5 min incubation at RT the sample was centrifuged (14.000rpm, 4°C) for 15 min. The aqueous phase was removed and transferred to a new reaction tube after adding 1μl of glycogen (Invitrogen). After brief mixing, 700μl of 70% (v/v in water) isopropanol was added and incubated for at least 20min at -20°C. Afterwards the sample was centrifuged again at 14.000rpm at 4°C for 20min. The supernatant was discarded and to the precipitate 1ml ethanol 80% (v/v in water) was added. After a final centrifugation step a 14.000rpm at 4°C for 5min, supernatant was removed again and the RNA pellet was dried at RT and resuspended in 12μl RNase-free water. Oligo dT primed RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) at 42°C for one hour.

PCR reactions for the amplification of immunoglobulin genes were performed using six forward primers (VH1RK-VH6RK) in combination with reverse primers either binding to the constant region of IgG or IgM. Each reaction was conducted in 50μl of 1x PCR buffer (AmpliTaq Gold), 2mM $MgCl_2$ containing 20mM dNTPs, 1,25μM primers, 1U of AmpliTaq Gold, and 1,0μl cDNA as template. Amplification was performed according to the following cycling conditions: after 6 min of denaturation at 94°C, 44 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min were performed, followed by a final extension at 72°C for 7 min.
For fluorescence labeling of the different J-families, four different fluorescently labeled J-primers (JH 1.2, JH 3, JH 4.5, and JH 6) were introduced by "run-off" PCR. One PCR reaction was performed in a volume of 30μl. This reaction mixture consisted of 1x PCR-Puffer (Ampli Taq), 0,4mM dNTPs, 1,2 U Ampli Taq Gold and 0,03mM primer. The PCR was performed according to the protocol: after 10 minutes of denaturation at 94°C, 4 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 10 min were performed. The "run-off" products were then separated on sequencing gels. Using automated DNA sequencer and the Immunoscope software, the size of peaks and their areas were visualized and analysed.

2.5 Laser microdissection of CD138 positive plasma cells

For laser microdissection an inverse Olympus fluorescence microscope (adapted to a laser microdissection unit (Molecular machines, Glattbrugg) was used. To remove cells from the tissue section a modified tube with a volume of 0.2ml was placed with its special adhesive lid onto area of interest and single infiltrating PCs were marked on the computer screen and automatically cut out by a UV laser beam. Laser velocity, focus and laser energy were adapted to optimal cutting performance. After cutting, the tube with the lid containing the single cell was removed from the cap holder. Then 25µl of buffer, consisting of 1x buffer, 2U/µl Prime RNase Inhibitor, was inverted and put immediately on dry ice.

2.6 Single-cell RT-PCR of microdissected plasma cells

Single laser-microdissected PCs were lysed in 25µl of RT-buffer containing 2U/µl RNase inhibitor. The appropriate volume of enzyme mix was added to the sample and transferred to 0,2ml PCR tubes (Eppendorf).

For the preparation of enzyme mix for amplification of IgG and IgM heavy-chain immunoglobulin genes or heavy- and light-chain immunoglobulin genes from one single cell, the following concentrations of components were used: 1x buffer containing 10mM dNTPs, 0,2µM of each forward primer and reverse primer, 2U/µl RNase inhibitor, and 1.2 (two reactions hc IgG and IgM) or 1.5 µl (three reactions hc IgG or IgM with lc) of enzyme mix. The primers for the amplification of Ig genes were used and modified according to (Carlos F.Barbas III et al., 2001).

For the amplification of IgG and IgM hc (or IgG hc and corresponding lc or IgM hc and corresponding lc) from single cells, 5µl (or 13µl for three reactions) of mix were added to the sample and distributed in 14µl (or 12µl) reaction volume to PCR tubes (0,2ml) containing prepared primer mixes. RT-PCR were performed with Eppendorf Mastercycler device according to the following cycling program: 50°C for 60 min, 95°C for 10 min, 45 cycles of 95°C for 30s, 58°C for 45s, and 72°C for 1 min were performed, followed by a final extension of 10 min at 72°C.

2.7 PCR product purification and sequencing

PCR products were separated by agarose gelelectrophoresis. Samples were loaded onto a agarose gel 1,2% (weight/volume) in TBE (Tris-Borate-EDTA) buffer pH 8 and performed at 180V for approximately one hour. After documentation (geldoc) PCR products of calculated size were cut out. Nucleic acids were purified from gel excisions using the MinElut PCR purification Kit from Qiagen.

Purified PCR products were adjusted to a concentration of 10ng DNA per μl, the appropriate reverse primer was added and sequenced at Synergene Biotech.

Sequence data from Ig genes were analyzed using DNA Star MegAlin, SeqMan, and IMGT online software tools.

2.8 Cloning and expression of antibodies

After sequence analysis of the first PCR product for Ig hc, Ig κc, or Ig λc, a second PCR reaction was performed with specific V region and J region primers containing restriction sites for cloning into expression vectors. Three different vectors were used for the expression of the hc IgG and the corresponding lc. The vector backbone contains a leader sequence, an human cytomegalovirus (HCMV) promoter region and an ampicillin resistance gene. Each vector additionally contains the constant regions for hc, κc or λc. The vectors and inserts were digested with AgeI and SalI (hc), BsiwI (kc), or XhoI (lc). After the ligation reaction and transformation of E. coli, clone sequences were compared to the first PCR product.
The eukaryotic cell line, 293T, grown in Dulbecco's Modified Eagle's Medium (DMEM; GibcoBRL) supplemented with 10% heat-inactivated ultralow IgG fetal calf serum (FCS) (Invitrogen) 100 μg/ml streptomycin, 100 U/ml penicillin G were seeded onto 150mm plates. At 80% confluency, cells were transfected using the calcium-phosphate precipitation method. Twelve hours post transfection the medium was replaced by serum-free D-MEM media supplemented with 1% Nutridoma SP (Roche). After 8 days in culture the supernatant was harvested and recombinant antibodies were purified by affinity chromatography using protein G sepharose (Amersham Bioscience). For a detailed description of recombinant antibody production see (Tiller et al., 2008).

2.9 Construction of a cDNA expression library

For the construction of recombinant cDNA libraries the Cloneminer Kit from Invitrogen was used. All procedures were performed according to the manufacturer's protocol.
In brief, poly A^+ RNA (BD Biosciences) from different CNS areas (whole brain, spinal cord, corpus callosum) and skeletal muscle was used. First-strand synthesis was performed by using SuperScript™ II reverse transcriptase (Invitrogen). mRNA was primed with an Biotin-attB2-oligo(dT) primer which hybridizes to the 3' mRNA poly(A) tail. After second-strand synthesis using E. coli DNA polymerase I and blunt-end ligation of the Gateway™ adapter molecule attB1 to the 5' end the cDNA was size-fractionated with exclusion of fragments smaller than 500bp by size-exclusion chromatography to remove truncated cDNA and non-ligated adapter molecules. The cDNA was flanked by attB sites (recognition sequences for lambda phage recombination enzymes) to circumvent, on the one hand, restriction digest and ligation and, on the other hand, to allow the recombination of the whole library cDNA inserts into an entry-vector for amplification. After plasmid purification the cDNA library was transferred via a second recombination reaction into an expression vector of choice. For quality analysis the number of primary clones was calculated, and single cDNA clones were sequenced (Figure 8).

Our intention was to induce transgene expression in mouse cell lines. After protein expression had occurred, the cells would have been stained with labeled recombinant antibodies and analyzed by FACS sorting. Positively stained cells would have been sorted into wells and regrown. To identify the expressed transgenes, the genomic DNA would have been extracted and a PCR using vector specific primers performed. The product would have been sequenced and should reveal the identity of the transgene expressed in this particular cell clone. In the case of a non-membrane-associated protein the cells could have been stained intracellularly. In this case, the cells cannot be regrown, so that a sufficient number of cells should be sorted (Figure 9).

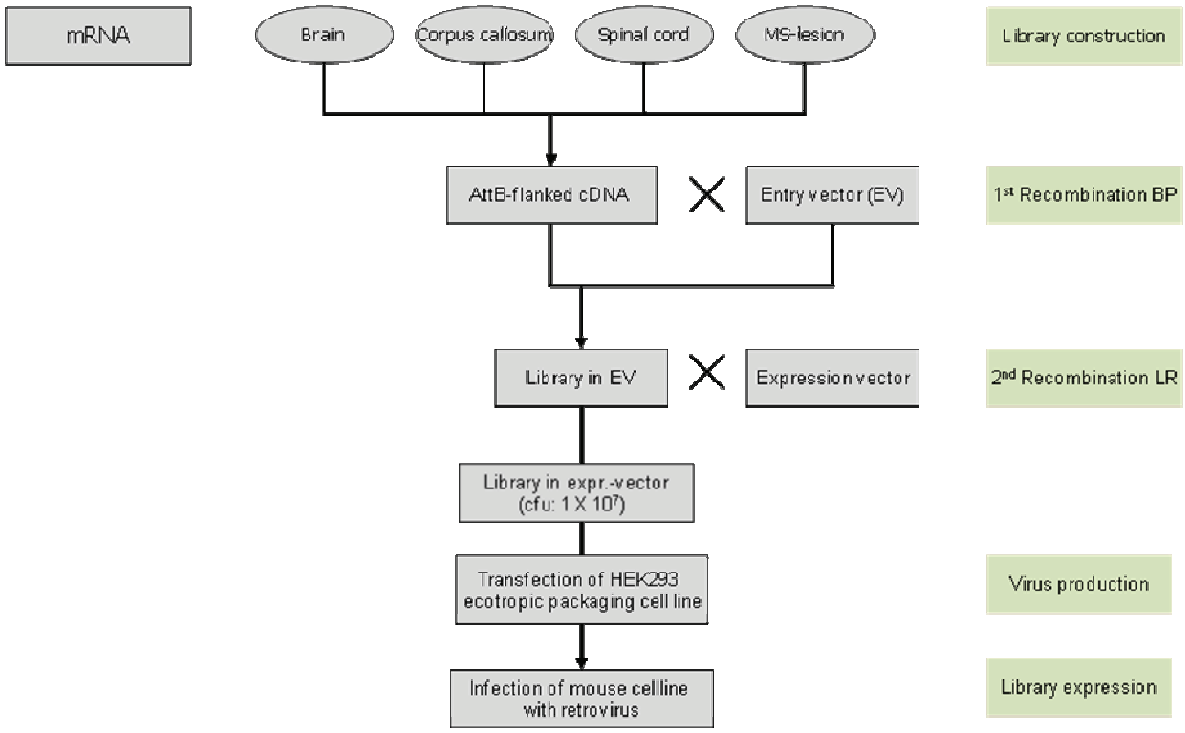


Figure 8. Principle of cDNA library construction.
This figure illustrates the general principle of the construction of a retroviral cDNA expression library. mRNA from different tissue sources is reverse transcribed and by a first recombination reaction recombined into an entry vector. In a second recombination reaction the cDNA library inserts are transferred into an retroviral expression vector. After amplification of the cDNA library in E. coli the plasmid DNA is used for the production of ecotrophic virus by transfection of a packaging cell line. After harvesting the viral supernatant, mouse cell lines can be used for infection.

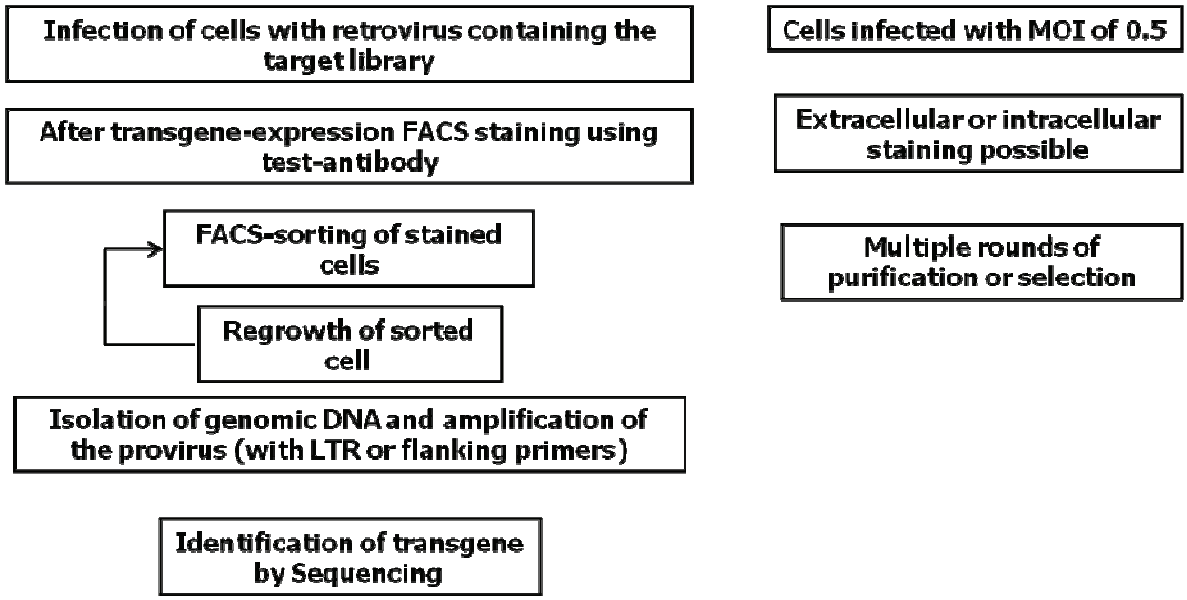


Figure 9. Principle of antigen identification.
This figure shows the strategy for antigen identification. Target cell lines are infected by retrovirus containing the cDNA library transcripts. After protein expression the infected cells are stained by the antibody, for which the antigen specificity will be determined. After FACS sorting of stained cells, the antigen can be determined by PCR of the proviral DNA and subsequent sequencing.

2.9.1 Retroviral expression vectors

As retroviral expression vector, pQCXIP was used from BD Bioscience. For transferring the cDNA library from the entry library vector into the expression vector, a Gateway™ recombination cassette was blunt-end ligated and cloned into the multicloning site of the vector. Transcripts which are inserted into pQCXIP vectors are bicistronically transcribed with an IRES-Puromycin sequence. As control vectors we recombined eGFP and MOG into the retroviral expression vector pQCXIP. eGFP was amplified from another vector by using specific primers with flanking attB sites. MOG was purchased already recombined in a Gateway entry vector (Invitrogen).

2.9.2 Virus production and retroviral infection of mammalian cells

After generation of a cDNA library from total human brain RNA, the cDNA was transferred to retroviral vectors as described, and retroviral packaging cell lines were transfected to generate mouse specific viral supernatant.

One day before transfection of the packaging cell line, 1.4×10^7 cells were seeded on a 150mm cell culture plate in 25ml media. After 80% confluency had been attained, cells were transfected by calcium-phosphate precipitation method. Therefore 50μg of retroviral plasmid DNA was diluted in 1mM Hepes solution. $CaCl_2$ was added to a final concentration of 250mM and a final volume of 750μl. After that, 750μl HeBS were dropwise added to the plasmid DNA and incubated for 10min at room temperature. The cells were transfected by adding the precipitate and then incubating for at least 6 hours. One day post transfection the exhausted media were replaced by fresh media. After 48 and 72 hours the retroviral supernatant was harvested centrifuged at 500g for 5min and the supernatant stored at -80°C. (For detailed information see Current Protocols in Immunology 10.17C)

3 Results:

3.1 Myositis tissues and histological analyses

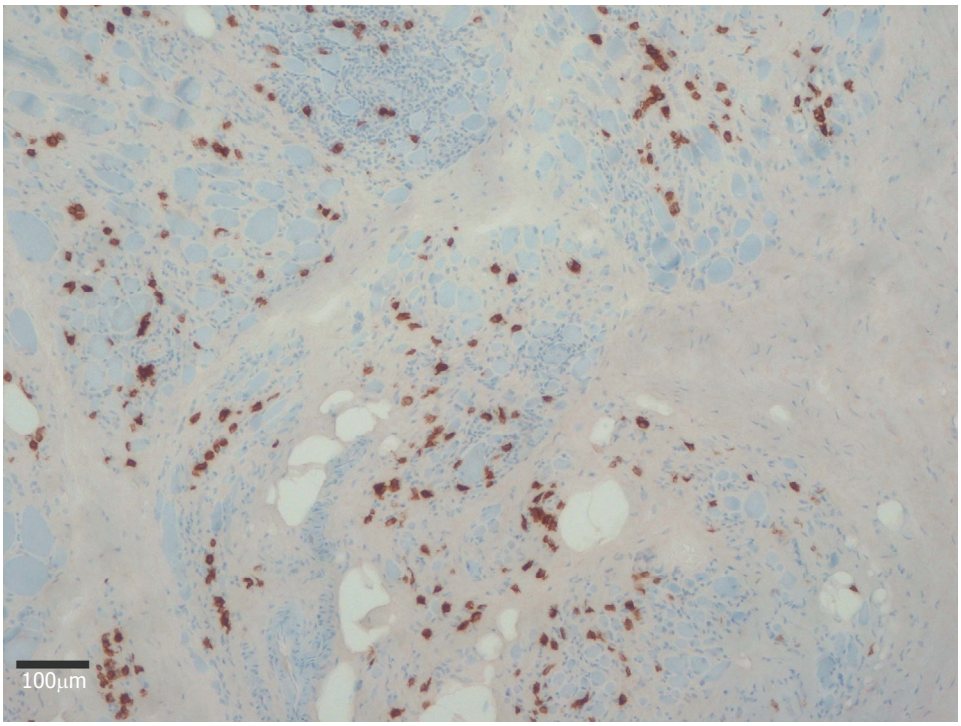


Figure 10. CD138$^+$ plasma cells in IBM 451/96.

The primary goal of our study was the analysis of the clonal composition of the PC repertoire in inflammatory myopathies (IM). Muscle biopsy specimens of patients with IM were obtained from the Friedrich Baur Institute, University Hospital Munich. For each patient, the diagnosis of an IM was established according to current clinical and histological criteria. Table 2 summarizes all important information concerning patient history and the results of the histological analysis characterizing the inflammatory infiltrate present in the biopsy. In most cases the obtained material was from the first performed biopsy. If the patient was treated with immunosuppessive agents at the time the biopsy was taken it is noted in the table. Creatine kinase (CK), an enzyme released from muscle fibers as a consequence of muscle destruction, is commonly used as a parameter for disease activity in IM. CK activity is listed in the biopsy summary table 2. We analyzed all biopsies by immunohistochemistry for the presence of B- and PCs. The histological analysis gave us information about the extent of inflammation and about the composition of the cellular infiltrate, as well. In most cases a medium to high infiltration by CD4$^+$ T cells and CD8$^+$ T cells was present. The presence of B

cells and/or PCs was found not to be a general feature of every IM biopsy. We could detect PCs in 2 of 6 PM, in 8 of 25 DM and in 7 of 10 IBM biopsies. In general, the number of PCs was higher than that of B cells. In PM only one $CD138^+$ biopsy also contained infiltrating $CD20^+$ B cells. In DM and IBM all $CD138^+$ biopsies also contained B cells in different quantities (Figure 10). To characterize the PCs further with respect to their Ig isotype, we performed stainings for three different isotypes IgG, IgM, IgA. IgG, and IgM were the prominent isotypes of the PCs; only in some cases we found additionally a low number of IgA-expressing PCs.

	Patients	CDR3-St	LM	Code	Age (y)/sex	Disease D.	Previous treat.	CK (U/μl)	CD3	CD4	CD8	CD20	CD68	CD138	IgG	IgM	IgA
1	**PM-01**	**x**	**x**	350/95	57/M	1y	none	238	**+**	**+ (5)**	**(nd)**	**-**	**-**	**+ (25)**	+ **(15)**	+ **(12)**	+ **(12)**
2	PM-02			308/98	39/F	6m	none	2318	**+**	**+**	**-**	**-**	**-**	**-**	**+**	**-**	**-**
3	PM-03			309/98	35/M	(nd)	(nd)	120	**-**	**+**	**-**	**-**	**+**	**-**	**-**	**-**	**-**
4	PM-04			394/98	55/F	(nd)	(nd)	(nd)	**+**	**+ (5)**	**-**	**-**	**+**	**-**	**-**	**-**	**-**
5	PM-05			215/98	62/F	1y	none	390	**+**	**-**	**-**	**-**	**(nd)**	**-**	**-**	**-**	**-**
6	**PM-06**	**x**	**x**	249/06	49/F	Sjögren Dis.	Steroide, MTX	290	**++**	**+**	**+ (5)**	**+ (15)**	**++**	**+ (175)**	**+ (25)**	**+ (100)**	**+ (12)**
7	**IBM-01**	**x**	**x**	451/96	62/M	2y	Steroide	300	**+++**	**+ (3)**	**+ (80)**	**+ (10)**	**+**	**+(100)**	**+ (50)**	**+ (3)**	**-**
8	**IBM-02**	**x**	**x**	373/96	70/F	1y	Steroide	296	**++**	**+ (5)**	**+ (4)**	**+ (6)**	**+**	**+ (35)**	**+ (20)**	**+**	**+ (5)**
9	**IBM-03**	**x**	**x**	379/96 S1	74/M	1y	Steroide, i.v. Ig	190	**++**	**+**	**+ (15)**	**+ (3)**	**-**	**+ (30)**	**+ (5)**	**+ (20)**	**-**
9	**IBM-03**	**x**	**x**	379/96 S2	74/M	1y	Steroide, i.v. Ig	190	**++**	**+**	**-**	**+ (4)**	**+**	**(nd)**	**-**	**-**	**-**
10	IBM-04			123/96	71/M	2y	none	200	**-**	**+ (5)**	**-**	**+ (8)**	**(nd)**	**-**	**-**	**-**	**-**
11	IBM-05			264/96	70/F	1y	none	190	**-**	**+ (5)**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
12	IBM-06	**x**		44/05	83/M	1,5y	none	240	**++**	**+**	**+ (25)**	**+ (20)**	**++**	**+ (75)**	**+ (20)**	**+ (30)**	**+ (3)**
13	**IBM-07**	**x**	**x**	323/06	64/M	2y	Steroide, i.v. Ig	363	**+**	**+**	**+ (10)**	**+ Clu (50)**	**+**	**+ (200)**	**+ (70)**	**+ (25)**	**+ (8)**
14	**IBM-08**	**x**	**x**	243/06	63/M	1y	none	489	**++**	**+**	**+ (2)**	**+ (15)**	**(+)**	**+ (70)**	**+ (35)**	**+ (10)**	**+ (5)**
15	IBM-09			415/06	66/M	7y	none	627	+	+	+ **(25)**	-	+	-	-	-	-
16	IBM-10			89/06	85/F	2y	none	1880	++	++	+ **(50)**	**+ (2)**	++	+ **(20)**	+ **(10)**	**+ (7)**	-
17	DM-01	**x**		353/95	54/F	6m	none	456	**(nd)**	**(nd)**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
18	DM-02			305/95	54/F	3m	none	411	**+**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
19	**DM-03**	**x**	**x**	289/95	35/M	3m	none	5920	**++**	**+ (10)**	**+ (15)**	**+ Clu (10)**	**++**	**+ (30)**	**+ (12)**	**+ (2)**	**+ (2)**
20	DM-04			4/96	52/F	(nd)	(nd)	1008..	**+**	**+**	**-**	**-**	**-**	**-**	**-**	**-**	**-**

Table 2a. Overview of all IM Biopsies.
(-) no cells detected; (+, ++, +++) cells detected in low, moderate, or high number; number in brackets means the number of positively stained cells, cell numbers refer to a normalized tissue area; (nd) not determined because of a bad tissue morphology or failed staining.

	Patients	CDR3-St	LM	Code	Age (y)/sex	Disease D.	Previous treat.	CK (U/µl)	CD3	CD4	CD8	CD20	CD68	CD138	IgG	IgM	IgA
21	DM-05			292/96	31/F	(nd)	(nd)	376	+	-	(nd)	-	-	-	-	-	-
22	DM-06			380/96	70/F	(nd)	(nd)	372	++	-	+	-	-	-	-	(-)	-
23	DM-07	x		439/97	35/M	6m	none	662	+++	-	+ (200)	+ Clu (60)	+	+ (60)	+ (40)	+ (30)	+ (3)
24	DM-08			273/98	54/M	(nd)	(nd)	410		0/+	-	-	+	-	-	(+)	
25	**DM-09**	x	x	354/98	54/F	3m	none	1008	+++	+	+ (12)	+ Clu (7)	+	+ (25)	+ (20)	(+)	(-)
26	DM-10			260/03	56/F	3m	none	5338									
27	**DM-11**	x	x	354/03	61/F	3m	none	1117	++	+ (25)	+ (4)	+ Clu (5)	+	+ (35)	0/+	+ (20)	+ (4)
28	DM-12			233/03	51/F	3m	none	172	+	+	-	-	-	-	-	-	-
29	DM-13			322/03	31/F	3m	none	130	+		-	-	-	-	-	-	-
30	DM-14			288/03	60/F	3m	none	310	-	+ (5)	-	-	-	-	-	-	-
31	DM-15			323/03						-	-	-	-	-	-	-	-
32	DM-16			253/04	77/F	6m	none	2883	+	+ (5)	-	+ (4)	-	-	-	-	-
33	DM-17			146/05	27/M	1m	none	2177		+	+ (20)	-	(+)	-	-	-	-
34	**DM-18**	x	x	14/06	49/M	1m	none	4103....	+	(nd)	(nd)	+ (5)	-	+ (40)	(nd)	(nd)	(nd)
35	DM-19	x		157/06	62F	1y	none	327	+	++	+ (20)	+ (5)	-/+	+/(5)	+	+	-
36	DM-20	x		91/06	31F	3m	none	260	+++	+++	+ (100)	+ (35)	+	+/(5)	+	+	-
37	DM-21	x		230/06	39F	3m	none	2979	++	+	+ (25)	+ (35)	-/+	+/(5)	+	(+)	-
38	DM-22	x		139/06	80/F	3m	none	548		++	+ (20)	+ (10)	++	-	+ (5)	(+)	-
39	DM-23	x		39/06	65/F	2y	Steroide, MTX	5879	-	+	+ (20)	-	-	+ (5)	-	(+)	-
40	DM-24	x		160/06	68/F	3m	none	5187	+	+	+ (8)	-	+	-	-	-	-
41	DM-25	x		118/06	66/m	2y	Steroide	100	++	+	+ (15)	-	-	-	-	-	-
42	HC			528/96	1/M	10d	none	210	-	+ (1)	-	-	-	+ (1)	-	-	-

Table 2b. Overview of all IM Biopsies.
(-) no cells detected; (+, ++, +++) cells detected in low, moderate, or high number; number in brackets means the number of positively stained cells, cell numbers refer to a normalized tissue area; (nd) not determined because of a bad tissue morphology or failed staining.

3.2 Identification of plasmablasts and terminally differentiated plasma cells

The first developmental change of GC B cells is the transient plasmablast state. These cells are CD138 positive, express high amounts of MHC class II, still divide, and secrete Ig in high amounts. Terminally differentiated PCs no longer divide and are characterized by low MHC class II expression. To determine the developmental state of the PCs in IM double-immunofluorescence stainings with an anti-CD138 and an anti-HLA-DR were performed to distinguish between plasmablasts and PCs. The presence of plasmablasts would indicate the presence of active GCs. In all IM, PCs, but not plasmablasts, were detected (Figure 11). This finding does not indicate the presence of active GCs with proliferating plasmablasts, however, we can not exclude the presence of such GCs in other areas of the inflamed muscle.

Additionally, we tried to detect GCs in the biopsy material by histological analysis. To identify GC B cells we performed a peanut agglutinin (PNA) staining. But, we could not detect any B cells which were stained by PNA (data not shown). The CD19 staining shows some infiltrating B cells (see table 2) and, in three tissues, cluster like accumulations of B cells, which are typical but do not provide conclusive evidence for the presence of GCs.

So far, we have no evidence for the presence of GCs in IM biopsies which have been used for PC repertoire analysis. In some biopsies clonal variations have been observed indicating ongoing affinity maturation.

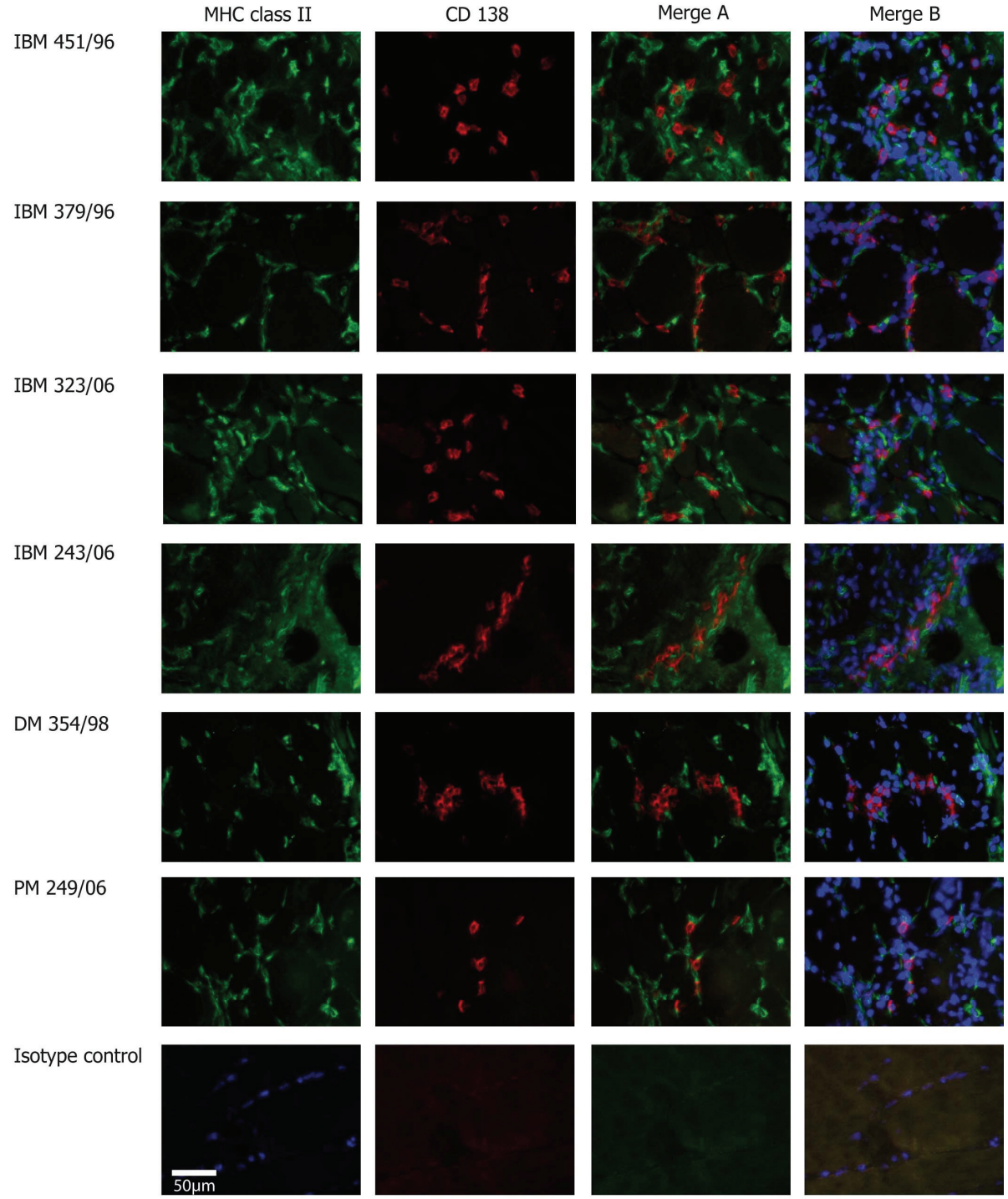


Figure 11. Immunofluorescence staining for the identification of plasmablasts and plasma cells. CD138 staining in red; HLA-DR staining in green. Merge A CD138 and HLA-DR; Merge B CD138, HLA-DR and Dapi The overlay of stainings for $CD138^+$ PCs and MHC class II shows no colocalization in any of the IM biopsies. This finding clearly shows that the $CD138^+$ cells are terminally differentiated PCs.

3.3 Characterization of the B-cell infiltrate by CDR3 spectatyping

After obtaining information about the extent of B lymphocyte infiltration and their Ig isotype, we were interested in overall clonal composition of the B- and plasma cells present in the tissue. Therefore, we analysed cDNA derived from several sections of the biopsies indicated in table 1, by complementarity determining region 3 (CDR3) spectratyping. This method allows the identification of clonally expanded B cells as individual "peaks" in the CDR3 length spectragram, whereas polyclonal populations give rise to a Gaussian-like distribution of the CDR3 spectragram. The basis of this method is an unbiased amplification of all heavy chain (VH) region genes present in the tissue. As an output of this method the distribution of the different V_H genes is given according to the length of their CDR3 region. The clonal composition is analysed at the V_H and J_H family levels. Spectratyping permits the detection of clonal composition with a resolution of each V_H and J_H Ig families. For more information on CDR3 spectratyping see (Pannetier et al., 1995). These spectratype experiments were done to complement LM as an independent technique for a clonal repertoire analysis Furthermore, we used this data to verify our data generated by LM. In the case of strongly expanded Ig populations it often is possible to directly determine the identity of the expanded clone by cycle sequencing. A strongly expanded clone detected in LM should be represented as one distinct peak in the spectragram and should also yield the same CDR3 sequence as that obtained from sc RT-PCR of excised PCs. The CDR3 spectratype analysis were performed in collaboration with the Max Planck Institute for Neurobiology in Munich.

As an example of a biopsy with a polyclonal hc repertoire represented as a Gaussian-like curve in the spectragram is DM 354/03 (Figure 12), an oligoclonal repertoire with distinct peaks is found in IBM 451/96 (Figure 13).

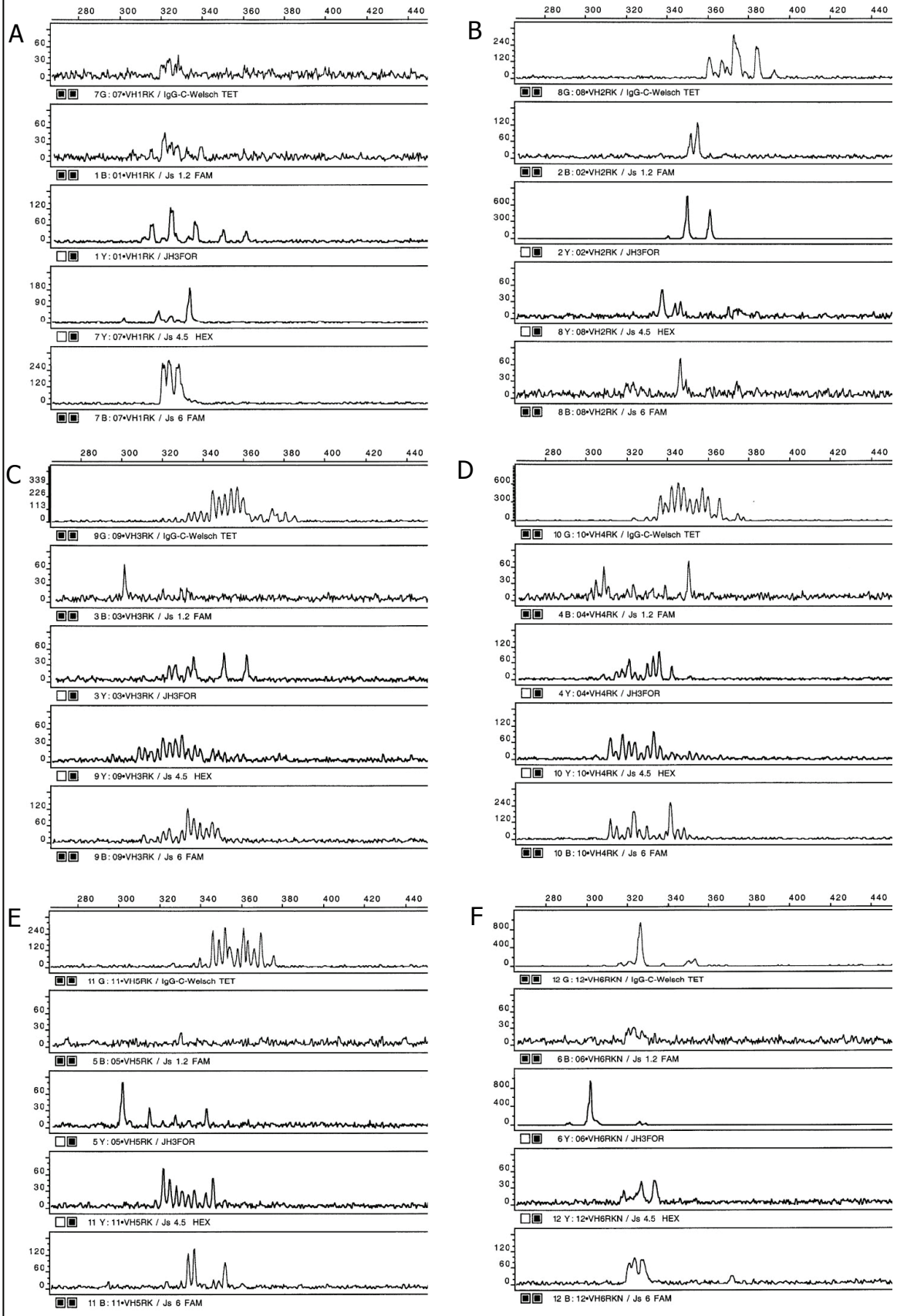


Figure 12. CDR3 spectragram of DM 354/03.
This figure shows the CDR3 length spectragrams for VH1-6 families. The peak height correlates to the relative abundance. The spectragrams for IgG are shown. In A the spectragrams for VH1 and the different JH families are shown (top down) VH1-constant region IgG, VH1-JH1,2, VH1-JH3, VH1-JH4, 5, VH6-JH6; In B the spectragrams for VH2 are shown (top down) VH2-IgG-C, etc.

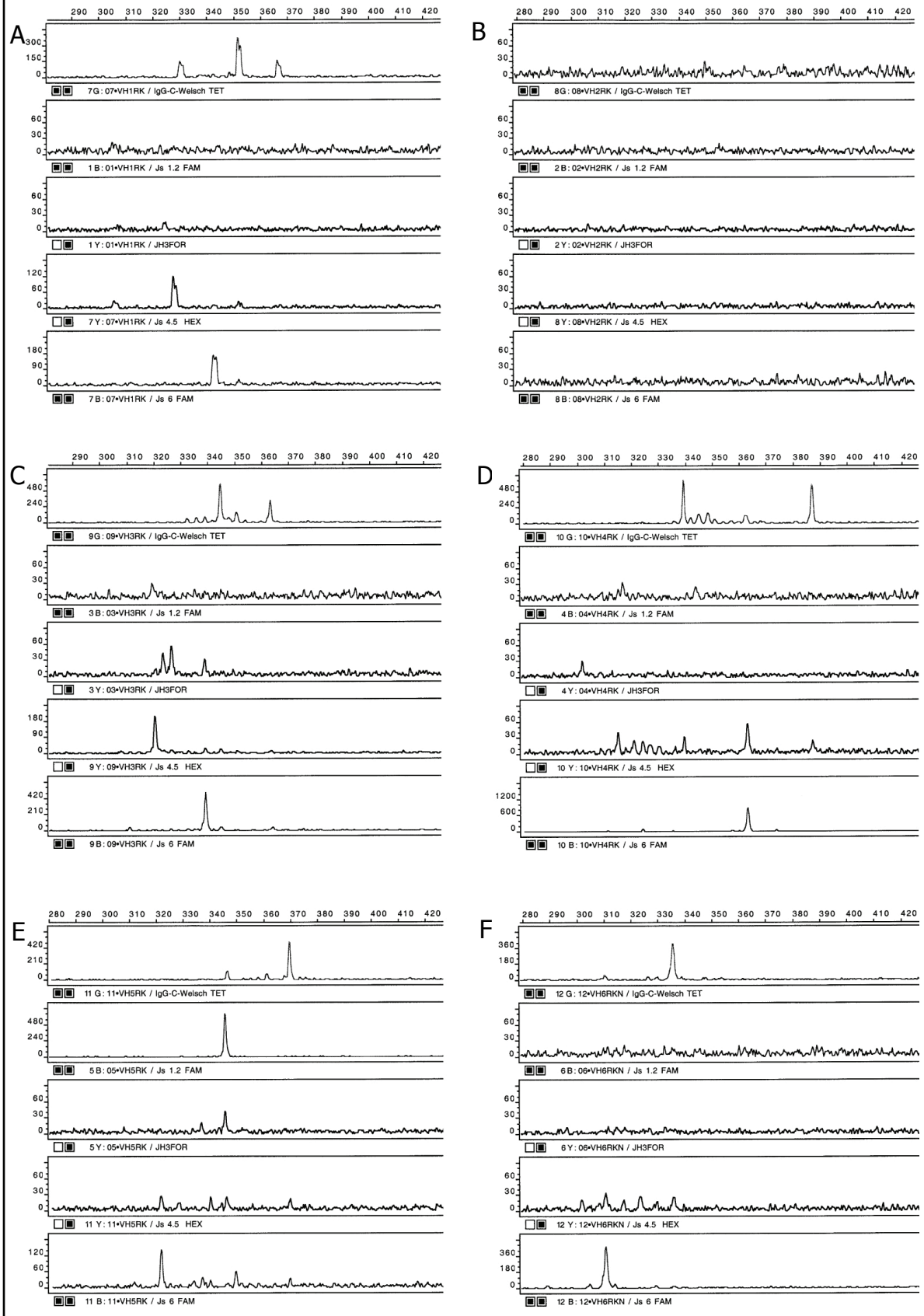


Figure 13. CDR3 spectragram of IBM 451/96.
The figure shows the CDR3 length spectragrams for VH1-6 families. The peak height correlates to the relative abundance. The spectragrams for IgG are shown. In A the spectragrams for VH1 and the different JH families are shown (top down) VH1-constant region IgG, VH1-JH1,2, VH1-JH3, VH1-JH4, 5, VH6-JH6; In B the spectragrams for VH2 are shown (top down) VH2-IgG-C, etc.

3.4 Characterization of the plasma-cell infiltrate by LM and sc RT-PCR

Our major goal in this project was the analysis of Ig gene rearrangements from infiltrating B- and PCs in diseased muscle from IM. Our intention was further the reconstruction of the original antigen specificity of antibodies secreted by these PCs. For this purpose, it was necessary to amplify both heavy and light chain Ig genes from individual cells, to maintain the right pairing of the chains, which is necessary for the reconstruction of the original antigen specificity by recombinant expression.

In our case, we were interested in the amplification of Ig transcripts from coding mRNA, because these transcripts are present in high quantities in PCs, and should serve as sufficient template for three different PCR reactions: heavy chain, light-chain kappa and light-chain lambda Ig.

A genomic approach was not followed because of only one coding sequence per cellular genome and additionally because of the presence of introns which would lead to a very long PCR product. There are some limitations when RNA is used instead of DNA. The first and most important limitation is the biological instability of RNA, which is based on the presence of RNA degrading enzymes in the cell itself and also in the environment. For this reason, the biological material (biopsy) has to be isolated from the patient under RNA preserving conditions. A second reason is that, after cutting the tissue into sections, the cellular compartmentalization is destroyed, and RNases may degrade the RNA. Therefore all further steps have to be performed under conditions in which the RNA integrity is well preserved. Additionally, RNA is more instable than DNA because of its chemical nature.

At the beginning of the thesis work it was therefore a major goal to establish a fast and efficient staining of immune cells, the dissection of single cells from stained dehydrated tissue sections and the amplification by RT-PCR of heavy- and light-chain immunoglobulin genes from single cells in which these conditions are maintained. For LM we used an inverted microscope with the device for dissecting tissue sections by UV laser. This microscope allows the efficient excision of cells in an acceptable time frame. The lid of a reaction tube was inverted over the area in which the PCs were present. After cutting, the lid was removed from the tissue area, and the cell remaining on the lid was put into buffer.

In the following procedures, after LM, the activity of RNases has to be inhibited during the lysis step as well as in the gene-specific reverse transcription of the RNA.

A lysis step is introduced to set free most of the RNA content from the fixed tissue section. We used tonsil tissue for establishing all steps. Because of the high risk of introducing DNA contamination, all steps were performed in separate rooms. RT-PCR reactions were set up in

a specially designated separate clean room with filtered air. Processing isolated PCs was the previous performed work before entering potential contaminated lab rooms. Special lab coats were weared to avoid cross-contamination. After establishing the method using tonsil tissue we had an efficacy of up to 60% for the RT-PCR reactions of single isolated PCs. Similar efficacies were reached for the reactions performed on PCs from IM biopsy samples. In a first LM experiment, RT-PCRs were set up for the amplification of hc transcripts of IgG and IgM isotypes. In further experiments, PCRs for the lc were performed, as well.

3.5 Summary of all LM experiments

After amplification of the heavy- and light-chain sequences of individual dissected cells, isolated products were sequenced. This allowed us to determine the CDR sequences, which are the highest variable sequence parts within the Ig gene and are responsible for antigen binding. In the sequence tables the experiment is indicated, the number given to each isolated PC and the usage of the V, D and J segments with the percentage of identity to the closest germline sequence (supplementary table page 85ff).

The CDR3 regions of hc and lc are mainly responsible for the antigen specificity of the (corresponding) antibody. The framework region serves as a scaffold for the three CDR regions. Table 4 summarizes all LM experiments performed with biopsies from all three IMs. In summary we dissected over one-thousand single PCs. The general efficacy of the PCR was in a range of 26.0% to 61.5%. From each biopsy we obtained 23 to 82 hc sequences of either IgG or IgM isotypes. The most prominent clonal expansions were found in three IBM tissues, especially in IBM 451/96 in which we detected eight expanded clones. Obtained expanded clones were verified by amplification of the same hc sequence in independent experiments performed at different times. In IBM approximately 40% of all amplified sequences belonged to expanded clones, whereas in DM and PM only 20% of all sequences where expanded. For detailed information on the nucleotide composition of all amplified hc sequences from isolated PCs see supplementary tables (page 85ff).

		Biopsy	No. of LM samples	No. of hc seq.	Efficacy of amplification (%)	Percentage of exp. sequences (%)	Rel. abundance of most exp. clone	Clonally expanded plasma cells
DM	4	DM-1 (14/06)	100	38	38.0	23.7	7,9	A (2) B (2) C (3) D (2)
		DM-2 (354/03)	100	62	62.0	22.6	4,8	A (2) B (2) C (2) D (2) E (3)
		DM-3 (289/95)	60	27	45.0	14.8	7,4	A (2) B (2)
		DM-4 (354/98)	70	36	52.0	22.2	5,5	A (2) B (2) C (2)
				163		x=20.65		
IBM	5	IBM-1 (451/96)	230	82	36.0	63.4	18,2	A (11) B (7) C (3) D (4) E (15) F (3) G (5) H (4)
		IBM-2 (373/96)	60	30	50.0	30.0	16,6	A (5) B (2) C (2)
		IBM-3 (379/96)	65	39	60.0	97.5	80,0	A (32) B (2) C (4)
		IBM-4 (323/06)	98	45	46.0	15.5	6,6	A (3) B (2) C (2)
		IBM-5 (243/06)	94	33	35,1	18.2	6,0	A (2) B (2) C (2)
				229		x=42.0		
PM	2	PM-1 (350/95)	110	32	29.0	31.25	6,3	A (2) B (2) C (2) D (2) E (2)
		PM-2 (249/06)	59	23	39.0	8.7	8,7	A (2)
				55		x=19.98		
			Σ 1046	Σ 450				

Table 4. Summary of all LM experiments.
In LM experiments four DM biopsies, five IBM biopsies and two PM biopsies were analyzed. The general efficacy of the RT-PCR of single PCs was between 29 and 62%. While in IBM tissues 451/96 and 379/96 strongly expanded clones were identified, in DM and PM more polyclonal sequence collections of low abundance were amplified. The clonal distribution is indicated as a capital letter for an expanded clone and in brackets the number for amplified identical sequences.

IBM 451/96

We isolated 230 PCs from this tissue. With a PCR efficacy of 36% we obtained 82 hc sequences of IgG isotype. PCRs for IgM heavy chain Ig were not performed because almost all PCs were of IgG isotype identified by IHC. The number of unique sequences was thirty-seven, from which eight were highly expanded. The percentage of expanded sequences were 54,3% of all amplified sequences; 45,6% of all sequences were not identified as expanded. All sequences contained SHM whether expanded or not. The expanded hc sequences were identical to each other at the nucleotide level. Only one clone showed two variants which were distinguishable by amino acid differences in the framework and CDR3 region.

IBM 379/96

Of 65 microdissected PCs, thirty-nine hc products could be amplified (efficacy: 60.0%). Seven sequences were of IgG isotype (17,9%). All other sequences were of IgM isotype. The sequence repertoire contained three expanded clones from which one was very highly expanded (32/40). The number of clones with different CDR3 region was five. All expanded sequences from individual clones were identical in their nucleotide sequence.

IBM 373/96

Twenty-eight hc products have been amplified from isolated cells from IBM 373/96. Twenty-two different clones were obtained from which three clones were clonally expanded and identical in their nucleotide sequence.

IBM 243/06

In three experiments we amplified 33 heavy-chain sequences. We identified two expanded clones, which were identical in sequence.

IBM 323/06

This fourth IBM biopsy comprises 45 hc sequences amplified from 98 dissected PCs (efficacy 46%). Three expanded clones, from which one clone had a quantity of three and two were found two times in the repertoire, were detected. Within these clones we found one clone which was identical to the secondly amplified sequence and two other clones showed variation in their sequence.

DM 14/06

From this DM biopsy 100 cells were dissected and 38 sequences amplified. The efficacy was, at 37%, somewhat low. Twenty-two sequences were of IgM isotype, and sixteen of IgG isotype. Two IgG clones and two IgM were both clonally expanded. One IgM and one IgG clone differed in several nucleotides from each other.

DM 289/95

From the second DM biopsy 27 hc sequences were amplified from 60 dissected samples (efficacy: 45%). From these sequences six were IgM, and twenty-one of IgG isotype. Two sequences were twice amplified and of identical sequence.

DM 354/03

Sixty-four sequences were amplified from 100 samples. We obtained 25 sequences of IgG and 39 of IgM isotype. In summary, we found five clones which were clonally expanded, two of them were identical, and three showed sequence variation.

DM 354/98

Seventy samples of the fourth DM biopsy were amplified and, with an efficacy of 52%, yielded 36 IgG sequences. We amplified three expanded and identical clones twice for each clone in the repertoire.

PM 350/95

From 110 dissected cells, 32 hc products were obtained. The efficacy was 29%. Most of the sequences were of IgG isotype. Five sequences were clonally expanded, and one of them showed clonal variation.

PM 249/06

From this biopsy, 59 samples were dissected and 22 heavy-chain sequences were obtained. One clone detected twice was expanded and identical in sequence.

3.6 Verification of expanded plasma-cell clones identified by sc RT-PCR

Since we analyzed the Ig hc sequence repertoire in IM by two independent methods, we were able to verify our results. LM and subsequent sc-PCR are very sensitive methods and are highly susceptible to producing artifacts. CDR3 spectratyping is not only suitable to reveal the distribution of the CDR3 length of Ig hc genes, but can also serve as the basis for sequencing of single peaks which represent expanded clones in the spectragram.

In both LM and CDR3 spectratyping experiments, we identified prominent clonal expansions in IBM 451/96 and IBM 379/96. We analysed the CDR spectragrams for peaks corresponding to expanded clones identified by sc-PCR and could amplify and sequence from CDR3 spectratype amplicons using family specific primers of the corresponding hc sequence. With this approach we verified two highly expanded clones of IgG isotype from IBM 451/96 and one IgM clone from IBM 379/96 (Figure 14 and 15). Compare also tables 5 and 6.

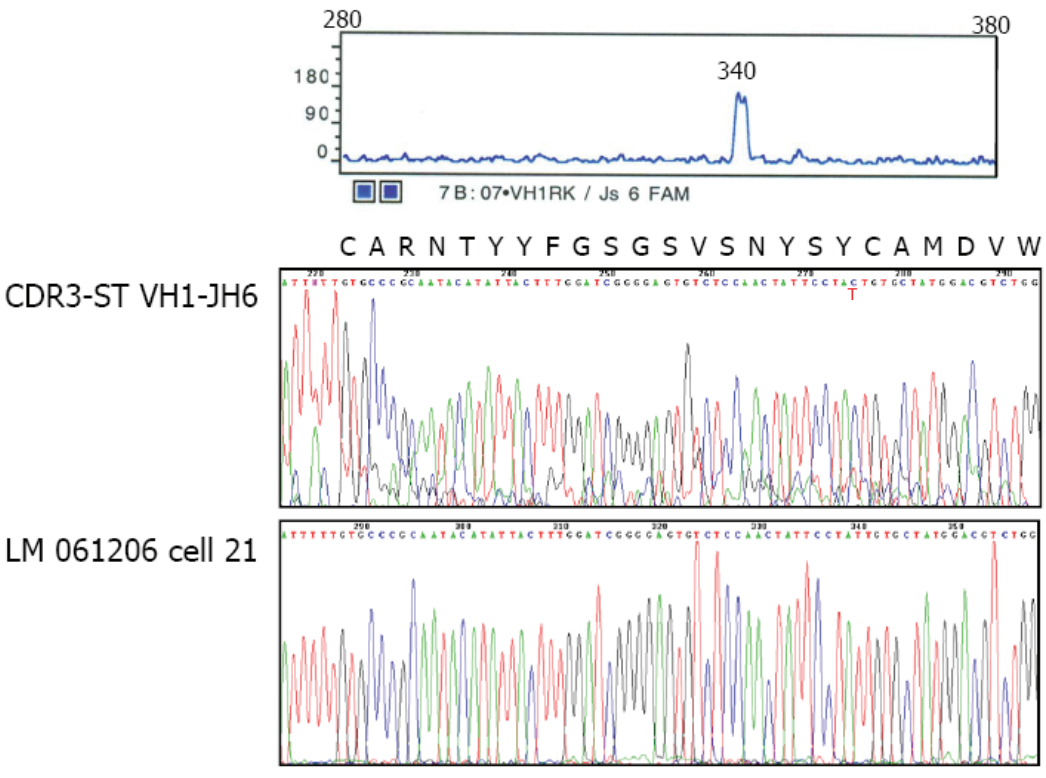


Figure 14a. Expanded clone from IBM 451/96
Upper panel: CDR3 spectragram with indicated VH and JH families; middle panel: sequence from amplified spectratype peak, above CDR3 aminoacid sequence; lower panel: sequence from LM with date of experiment and cell number.

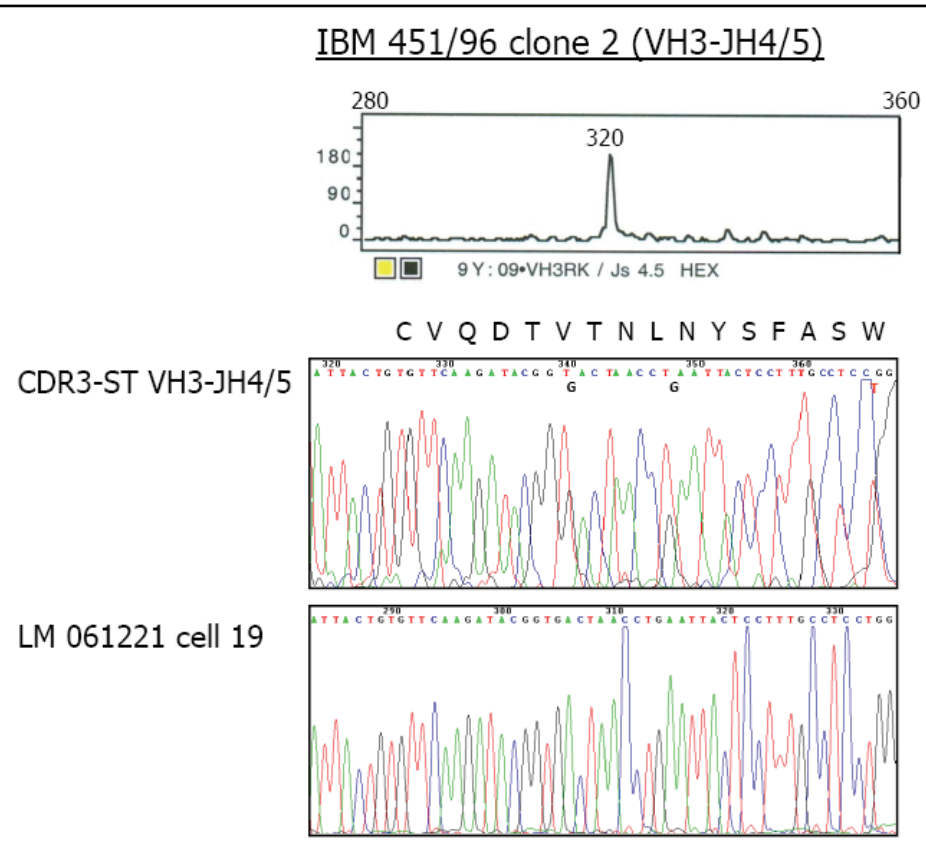


Figure 14b. Expanded clone from IBM 451/96

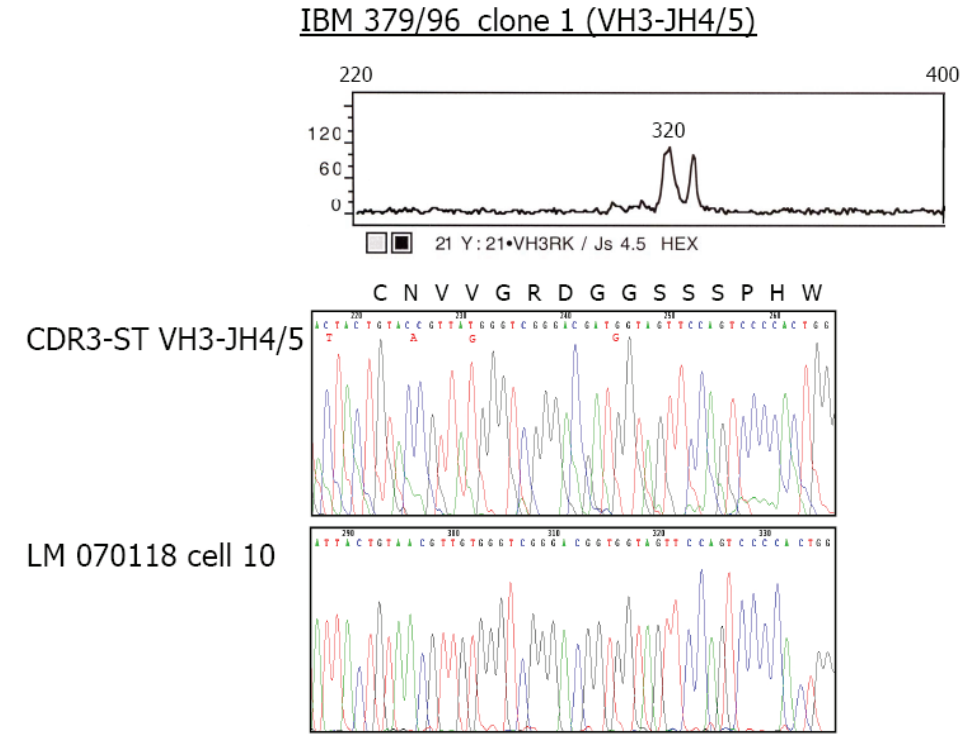


Figure 15. Expanded clone from IBM 379/96.
Upper panel: CDR3 spectragram with indicated VH and JH families; middle panel: sequence from amplified spectratype peak, above CDR3 aminoacid sequence; lower panel: sequence from LM with date of experiment and cell number.

3.7 Clonal variation of expanded plasma-cell clones

During B-cell maturation the sequence of the Ig genes is modified by the process of affinity maturation. This reaction, which under normal physiological conditions takes place in a GC, involves a selection process for the highest affinity of the antibody to its antigen. The sequence of the CDR3 and framework regions is mutated by the activity of AID, which induces SHM and also class switch recombination (Muramatsu et al., 2000). This reaction generates a population of mutated clones which are related in sequence but differ in the number of mutations.

In our Ig sequence collection from PCs isolated from IM biopsies we have identified eleven cases of clonal variation. This phenomenon was present in five biopsies (DM 354/03 n=3, DM 14/06 n=3; IBM 323/06 n=2, IBM 451/96 n=1; PM 350/95 n=1). In one clonal variation group, two to three related sequences were present. The occurrence of such variation suggests that these cells underwent a GC reaction which is antigen-driven. In figure 8, one example of a clonal variation is shown. Differences in the mutational pattern between the clones allow the reconstruction of the developmental steps of the clonal variation family members. An interesting finding in this context was that the expanded clones in IBM 451/96 were with one exception completely identical in sequence. In one clonal variant group, two very similar but not identical clones were found and are shown in figure 16. In comparison to the closest germline sequence, cell 3/061212 is more similar than are cells 22/070521 and 23/070521. The precursor of cells 22 and 23 is cell 3 which sequence was found twelve times in isolated PCs. The mutations are mainly located in the CDRs.

The alignment shown in figure 17 contains three completely different mutational patterns. One can only determine the distance from the closest germline sequence, but cannot bring the sequences in relation to each other, because they do not share any mutations and clones which reflect their relationship were not amplified.

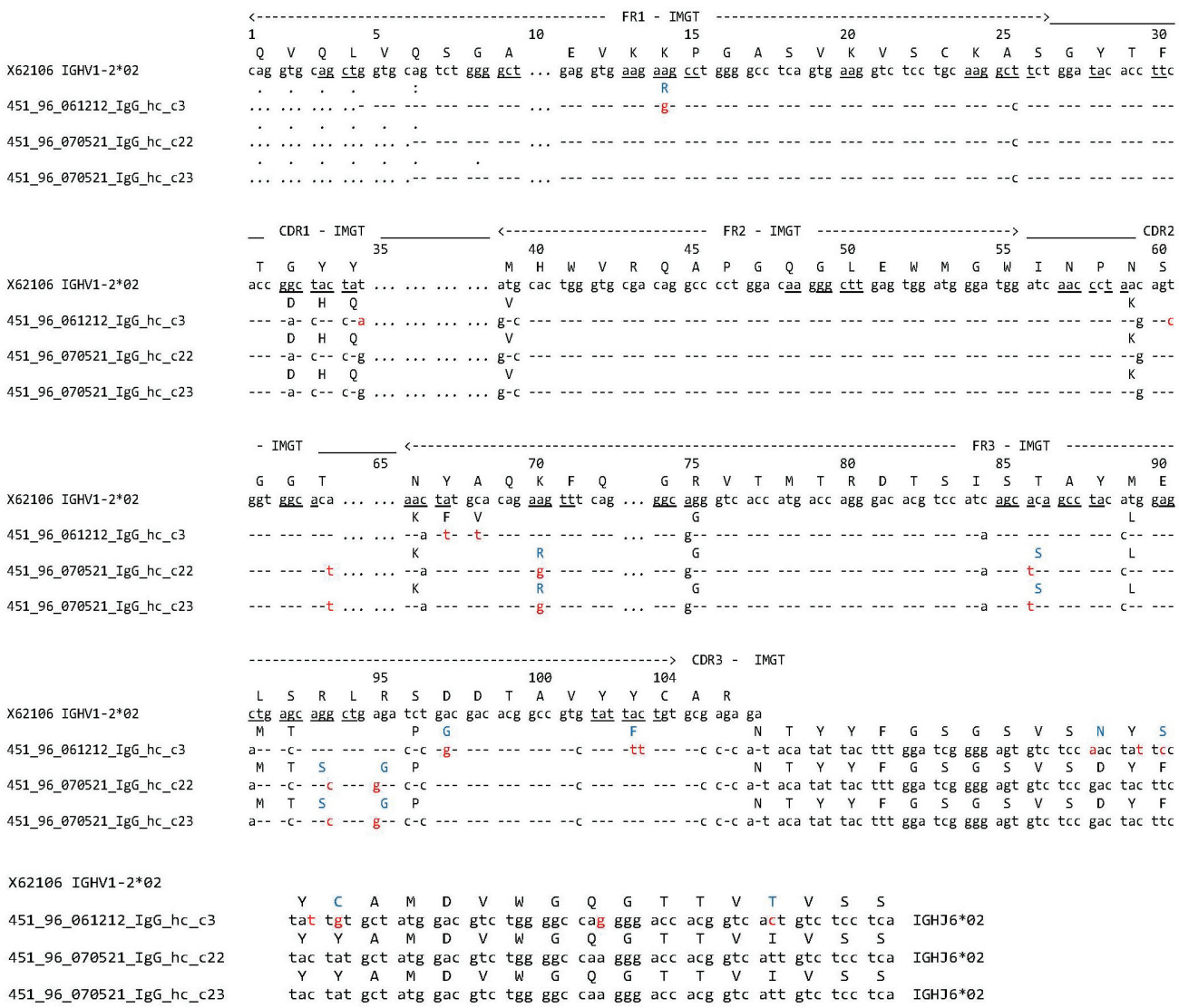


Figure 16. Alignment of clonal variants from IBM 451/96.
Upper panel: Alignment of nucleotide sequences with comparison to the closest germline sequence (-): identical nucleotide; replacement mutations are indicated.

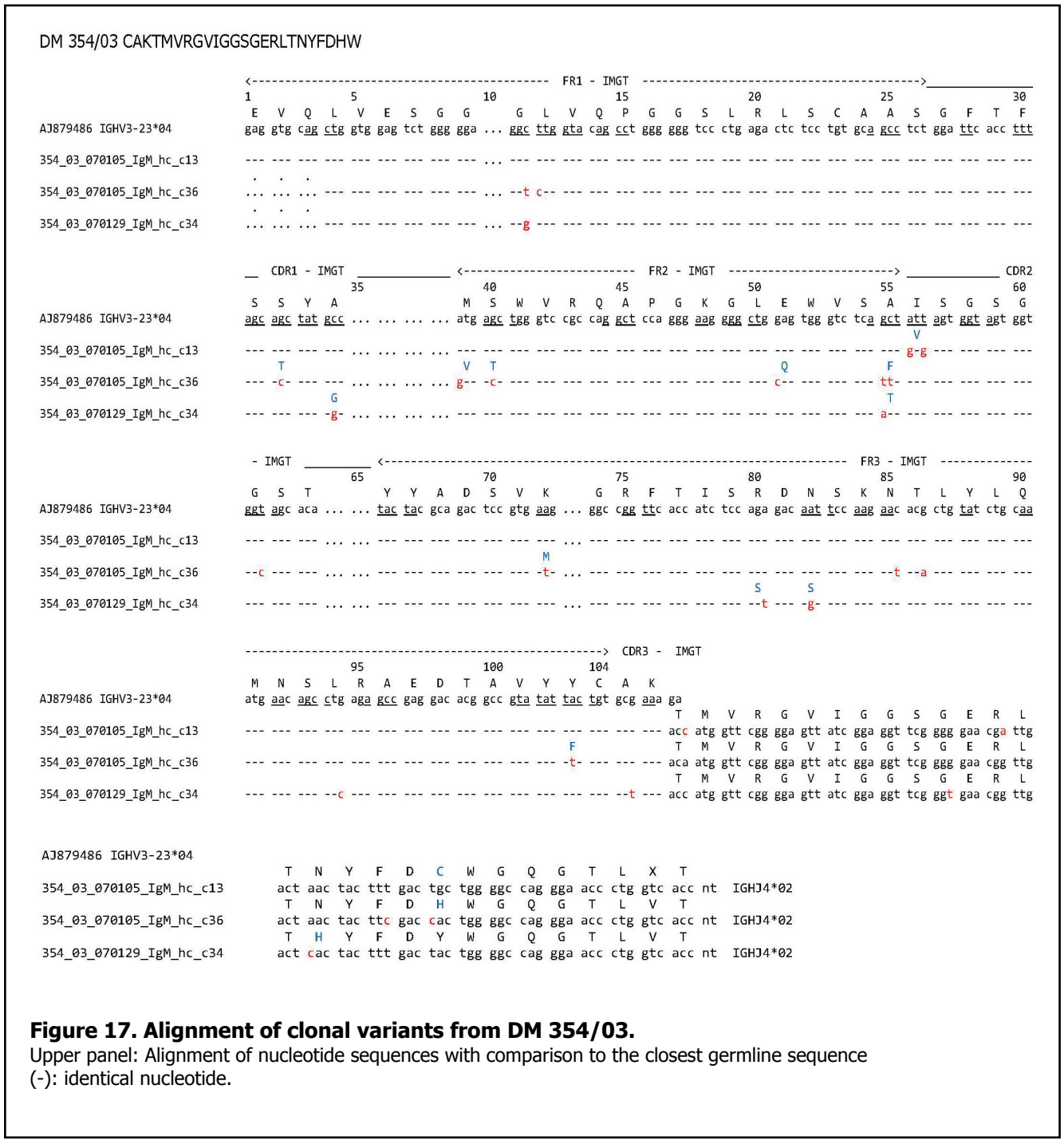


Figure 17. Alignment of clonal variants from DM 354/03.
Upper panel: Alignment of nucleotide sequences with comparison to the closest germline sequence (-): identical nucleotide.

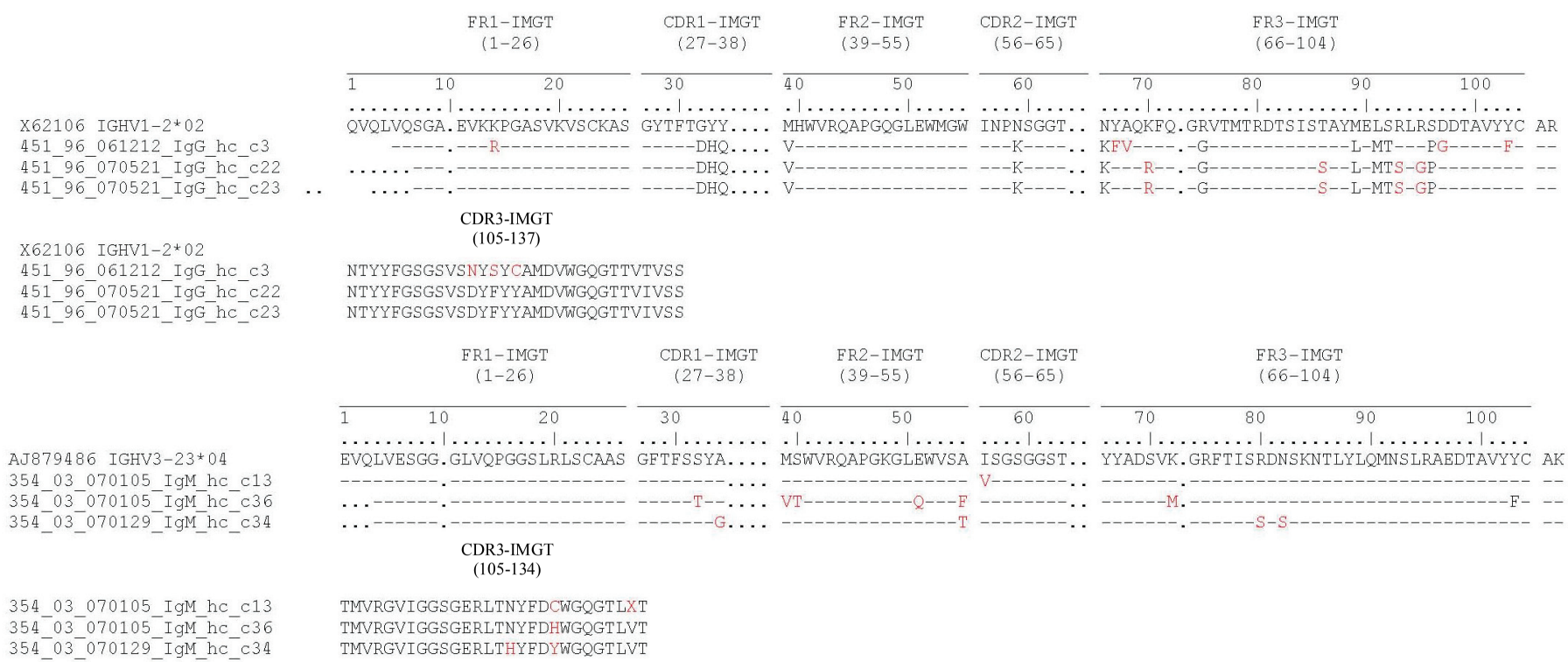


Figure 18. Alignment of clonal variants from IBM 451/96 and DM 354/03.
Upper panel: Alignment of the amino acid sequence with comparison to the closest germline sequence for IBM 451/96
Lower panel: Alignment of the amino acid sequence with comparison to the closest germline sequence for DM 354/03
(-): identical amino acid, not-shared replacement mutations are shown in red

3.8 Mutational analysis

In peripheral lymphoid organs, further processes take place to increase the affinity of the antibody to its antigen, which is called affinity maturation. Mutations which are introduced in the CDRs could have an effect on the antigen specificity. If mutations within the sequence result in an amino acid change they are called "replacement mutations" (R), if the amino acid sequence is not changed, they are called "silent mutations" (S). Only replacement mutations might have an effect on the affinity when they target the CDRs. Therefore, the mutation pattern and nature (replacement vs. silent) are a fingerprint of affinity maturation, and its analysis gives us important information about the function of the PCs in IM.

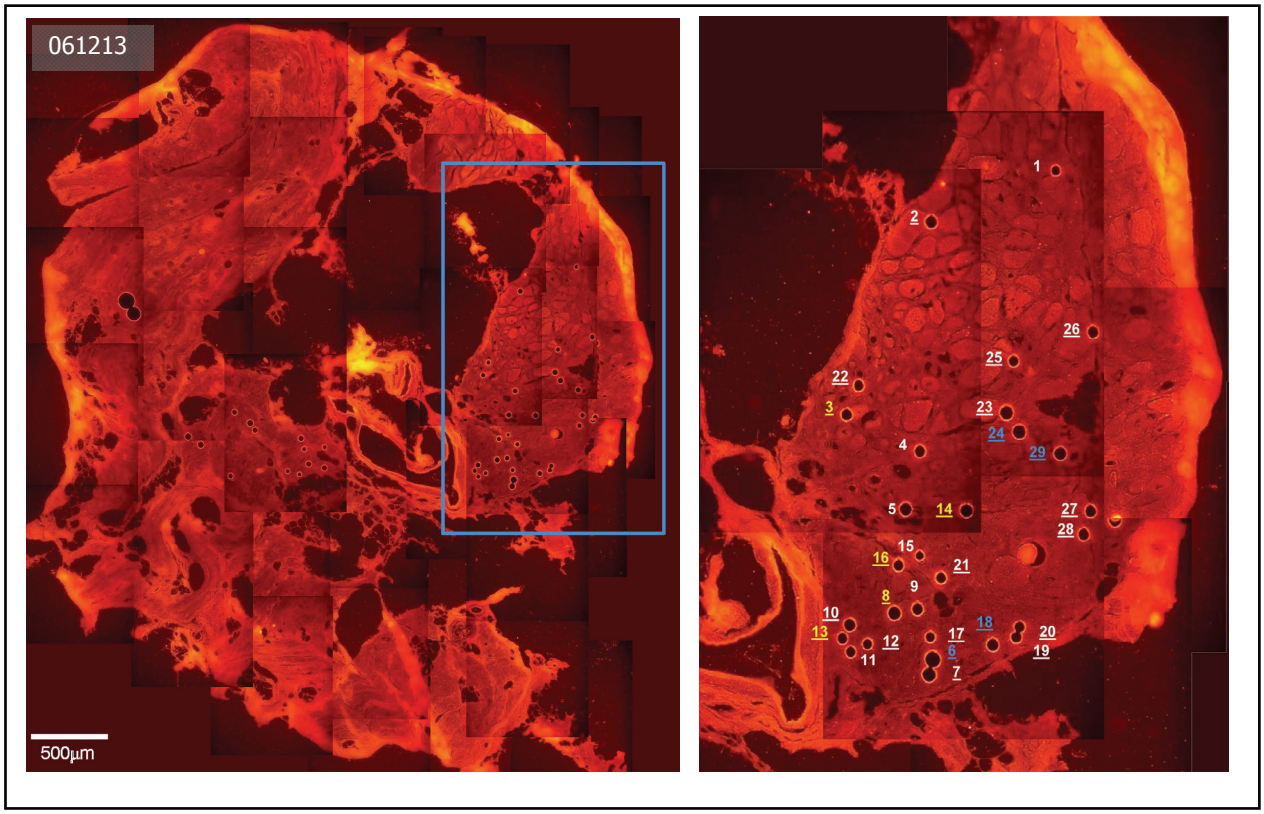
We analyzed the mutational distribution within the V_H sequence, as well as its frequency and also its character in the sequence collection of IBM 451/96. By comparison of the ratio of replacement to silent mutations in CDR regions and framework regions from clonally expanded and polyclonal sequences, we can assume that an antigen-driven process took place (Qin et al., 1998). We analyzed the R and S mutations in hc sequences from IBM 451/96. We compared the ratio of R- versus S- mutations within the expanded sequence group and the single occurring sequences. We found the ratio of R/S mutations in the framework and the CDRs is higher in expanded clones than in polyclonal sequences (Table 5). These findings further support an antigen-driven humoral immune response in IBM 451/96.

	non exp. clones	exp. clones
Mut R/S (Fr)	2.4	4.6
Mut R/S (CDR)	3.6	7.0

Table 5. Ratio of replacement versus silent mutations (R/S) in Framework 1-3 and CDR1-3 from plasma cells isolated from IBM 451/96.

3.9 Topologies

Because of the fact that each cell is individually isolated by LM we not only have the information of the expressed Ig gene of a each cell, but also know its location within the tissue, as well. We are, therefore, able to reconstruct the immune cell topology at the time when the biopsy was performed. In the case of IBM 451/96, we determined eight highly expanded clones together with their positions in the tissue sections. We can conclude from this topology established over six independent experiments, that the clonally expanded PCs with identical Ig gene are not randomly distributed, but are preferentially found in distinct areas of the tissue section. When we divide the section into quadrants we can determine locations in which the different expanded clones are present. By performing several LMs of adjacent sections, we can show clones in the same location, which verifies our finding. For example, clone 1 (yellow) and clone 2 (blue) are mainly found in quadrant 1, whereas clone 5 (green) is present in quadrant 3. Please compare LM 061212 with LM 061221, 070116 (Figure 19).



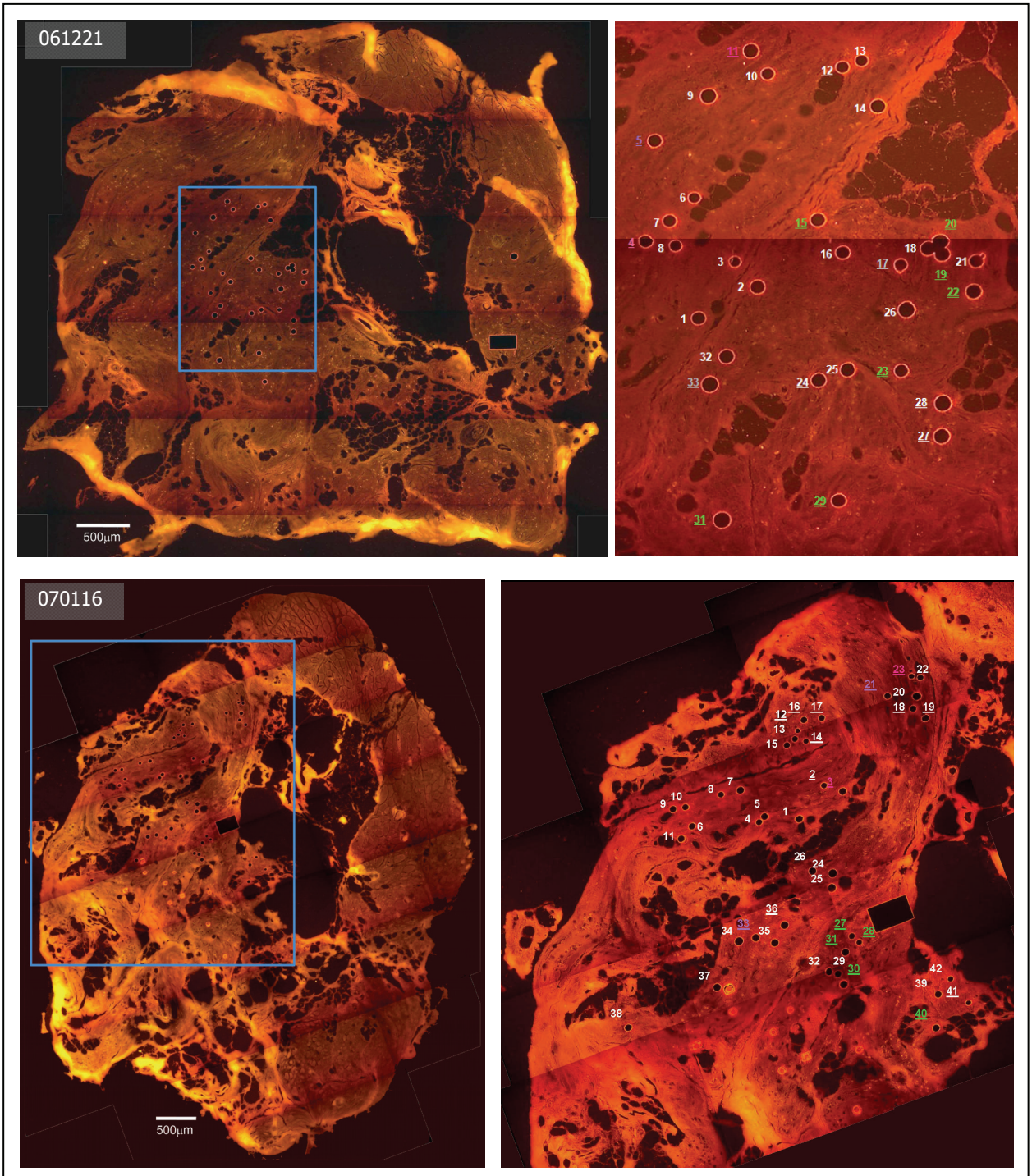


Figure 19. Overview of three LM experiments.
The left pictures show an overview of the tissue section used for LM. The detailed view shows the positions of the isolated plasma cells. The numbers in yellow and blue designate excised cells belonging to the same clone. White numbers not underlined indicate cells which did not yield a PCR product. White numbers underlined represent PCs with unique sequences.

3.10 Cloning and expression of antibodies

After having amplified the hc and lc sequences from several expanded PC clones from IBM 451/96 and 379/96, we started to clone the PCR fragments into eukaryotic expression vectors. See expanded clones from IBM 451/96 with corresponding light-chain amino acid sequence listed below (Table 6) and expanded clones from IBM 379/96 (Table 7). The recombinant antibodies are currently being produced in mammalian cells, as described above. These antibodies will be used for experiments to identifiy their corresponding target antigen.

Clone		CDR3 amino acid sequence	V	D	J	Frequency
1	hc	**C A R N T Y Y F G S G S V S N Y S Y C A M D V W**	V1-2*02	D3-10*01	J6*02	11/81
	lc	C Q Q Y G S P P P T T F	V3-20*01	n. a.	J5*01	
2	hc	**C A R D E S S S N K S R R R R F D P W**	V1-18*01	D3-22*01	J5*02	7/81
	lc	n. d.				
3	hc	**C A R G K R G A R D N Y F D Y W**	V1-18*01	D1-14*01	J4*02	3/81
	lc	n. d.				
4	hc	**C A R R G R L D S T S R F Y F D F W**	V3-74*01	D6-19*01	J4*02	4/81
	lc	C S S H A G G D N G Y V L F	V2-11*02	n. a.	J2*01	
5	hc	**C V Q D T V T N L N Y S F A S W**	V3-9*01	D4-17*01	J4*02	15/81
	lc	C S S Y I T S T T L E G H V V F	V2-14*01	n. a.	J2*01	
6	hc	**C A K C G P F G G V M V I P F D Y W**	V3-23*04	D3-16*01	J4*02	4/81
	lc	C Q Q Y H T L P P L T F	V1-33	n. a.	J4*01	
7	hc	**C V R D G Y C T S A N C Y F F Q H W**	V3-33*01	D6-13*01	J4*02	5/81
	lc	C Q Q G F S P P L T F	V1-39*01	n. a.	J4*01	
8	hc	**C A R N S H G G R S S D A F D I R**	V3-33*01	D1-26*01	J3*02	3/81
	lc	C Q Q F N S Y P R T F	V1-13*02	n. a.	J5*01	

Table 6. CDR3-sequences of clonally expanded PCs from IBM 451/96.
The table lists all expanded heavy chain sequences from IBM 451/96 and if determined the light chain sequence as well. The different V, D and J families are listed for every clone and the quantity of each clone as well. Underlined numbers indicate that the corresponding antibody currently being expressed. Colored numbers refers to figure 19 indicating expanded clones.

Clone		CDR3 amino acid sequence	V	D	J	Frequency
1	hc	**C N V V G R D G G S S S P H W**	V3-15*01	D4-23*01	J4*02	32/40
	lc	C Q Q Y Y T T L L T F	V4-1*01	n. a.	J4*01	
2	hc	**C A R G V C S G G T C L G Y W**	V3-74*02	D2-15*01	J4*02	2/40
	lc	C H Q Y Y N T P F T F	V4-1*01	n. a.	J3*01	
3	hc	**C A R E A P E G D C T T I S C Y R Y N G M D V W**	V1-69*01	D6-13*01	J6*02	4/40
	lc	n. d.				

Table 7. CDR3-sequences of clonally expanded PCs from IBM 379/96.

3.11 Construction of a mammalian cDNA expression library

We generated size-fractionated, non-normalized, non-full length selected cDNA libraries. During cDNA library construction small DNA fragments (<500bp) were strongly reduced by size-exclusion chromatography. The cDNA libraries were not normalized to reduce the amount of highly expressed genes and increase that of low expressed transcripts.

The number of clones prior to library amplification was at least 0.5-1.0 x 10^7 colony forming units (cfu) as determined by plating out E. coli containing cDNA in serial dilutions. The primary library was maintained in a cloning plasmid for maintenance in bacteria (E. coli). Since we attached attB sites (recognition sequences for lambda phage recombination enzymes) to the cDNA, the whole library could be recombined into an entry-vector for amplification and via a second recombination reaction into an expression vector of choice. By performing recombination reactions the need of using restriction enzymes is avoided, which might have cut within the cDNA transcript.

As an expression vector the retroviral vector pQCXIP was used and modified by insertion of a Gateway recombination cassette. The transgenes are bicistronically transcribed via an internal ribosome entry site (IRES) puromycin selection marker. This allows the selection of infected cells.

The human brain library recombined into the expression vector was amplified using 280 x 150mm LB plates for generating plasmid DNA for the transfection of the packaging cell line. As packaging cell line the high titer ecotrophic retrovirus producing cell line 293T Phönix Eco was used (Pear et al., 1993), which has been stably transfected with a Moloney Gag-Pol-IRES-Lyt2 construct. These cells were then stably transfected with Moloney ecotropic envelope gene (see Materials and Methods). The expression vector contains only the packaging signal sequence and the Long terminal repeats (LTR) for the insertion into the mammalian genome.

The main advantage of a retrovirally mediated gene transfer is the stable integration of the transgene (proviral DNA including the Long terminal repeats (LTR)) into the genome and the control of number of integrated sequences per cell, by adjusting the multiplicity of infection to 0.5 per cell. These advantages make the transgene analysis of cells more feasible compared to transient transfections, in which the copy number cannot be adapted and transgene expression will decrease over time. Transient transfections of cells are easier to perform, but the high copynumber of vector molecules per cell (20-100) makes identification of the transgene impossible.We have generated several different libraries. As an example,

the results of one cDNA library constructed from whole brain RNA is shown below. The number of primary clones was 1.0 x 10^7 cfu.

For the verification of a successful and effective recombination reaction restriction digests were performed. The amount of clones containing a cDNA insert was above 90% (Figure 20).

From a limited number of clones, sequence reactions were performed to determine brain specific cDNAs and the presence of full-length open-reading frames (Table 8).

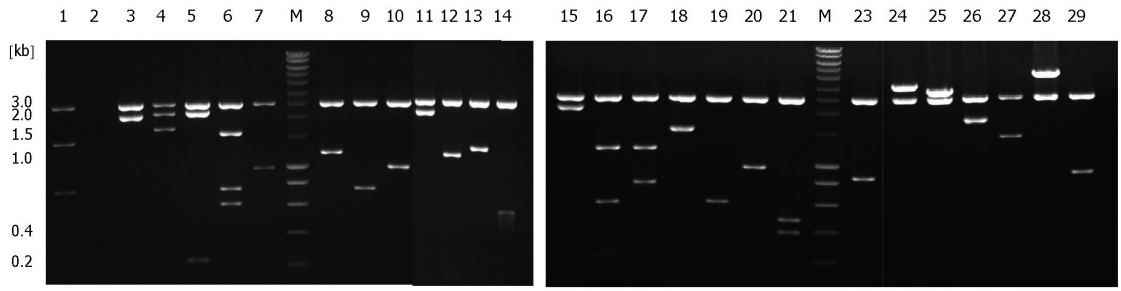


Figure 20. Restriction digest of cDNA library clones.
Restriction digest of plasmid DNA from cDNA library clones. As restriction enzyme BsrGI was used, which cut 5' and 3' of the multicloning site. The digested empty vector is visible in lane 1.

Sample Name	Size gel (kb)	Identity	Size Blast (bp)	Gen Bank	Full Length
Lib sc 01	2	Myelin basic protein	2156	BC047521	no
Lib sc 02	3.6	Colony stimulating factor Rezeptor	3904	BC047521	yes
Lib sc 03	2	Ras related small GTPase	2042	BK001232	yes
Lib sc 04	3	Proteolipid-protein	2777	M27110	yes
Lib sc 09	2	Myelin basic protein	2156	BC047521	yes
Lib sc 12	0.5	nd	na	na	na
Lib sc 13	2	myelin basic protein	2156	BC047521	yes
Lib sc 14	2.1	Tyrosine kinase receptor (trk-e)	3554	X74949	no
Lib sc 15	2.1	unknown	na	BAC RP13-82066	na
Lib sc 21	3	Acetyl-coenzyme A dehydrogenase	2250	BC000399.2	yes
Lib sc 22	2.8	Glial fibrillaric acid protein	3035	NM002055	no
Lib sc 25	4	KIAA0747	4026	AB018290	yes

Table 8. Results of sequence analysis of cDNA library clones.
In this table the size of the cDNA insert, as well as the blast result with the corresponding size according to the Gene Bank entry is shown; the last column provides information about the length of the transcript sequence.

As positive control vector constructs, the cDNA transcript of eGFP and MOG was recombined into the same retroviral expression vector as for the human brain cDNA library expression. Transgene expression was analyzed after puromycin selection of the infected mouse cell line. As expected almost all cells were infected with either eGFP or MOG (Figure 21A). These constructs were important controls for the functionality of the expression system. They served as control constructs during production of retroviral particles containing library cDNAs and infection experiments with library virus. However these control experiments are of limited value, because cells have been infected with only one virus containing either eGFP or MOG.

To show the functionality of the retrovirally transferred cDNA library, we infected mouse cells with the human brain cDNA library and tried to detect by FACS MOG expression using the same antibody as in previously performed control experiments (Figure 21B). The FACS plot shows no differences for the control staining compared to the MOG specific staining. According to these experiments and other assays we were not able to show cDNA library expression in mammalian cells. Because of these difficulties and the high effort to construct and validate the library approach, working on that project was not continued.

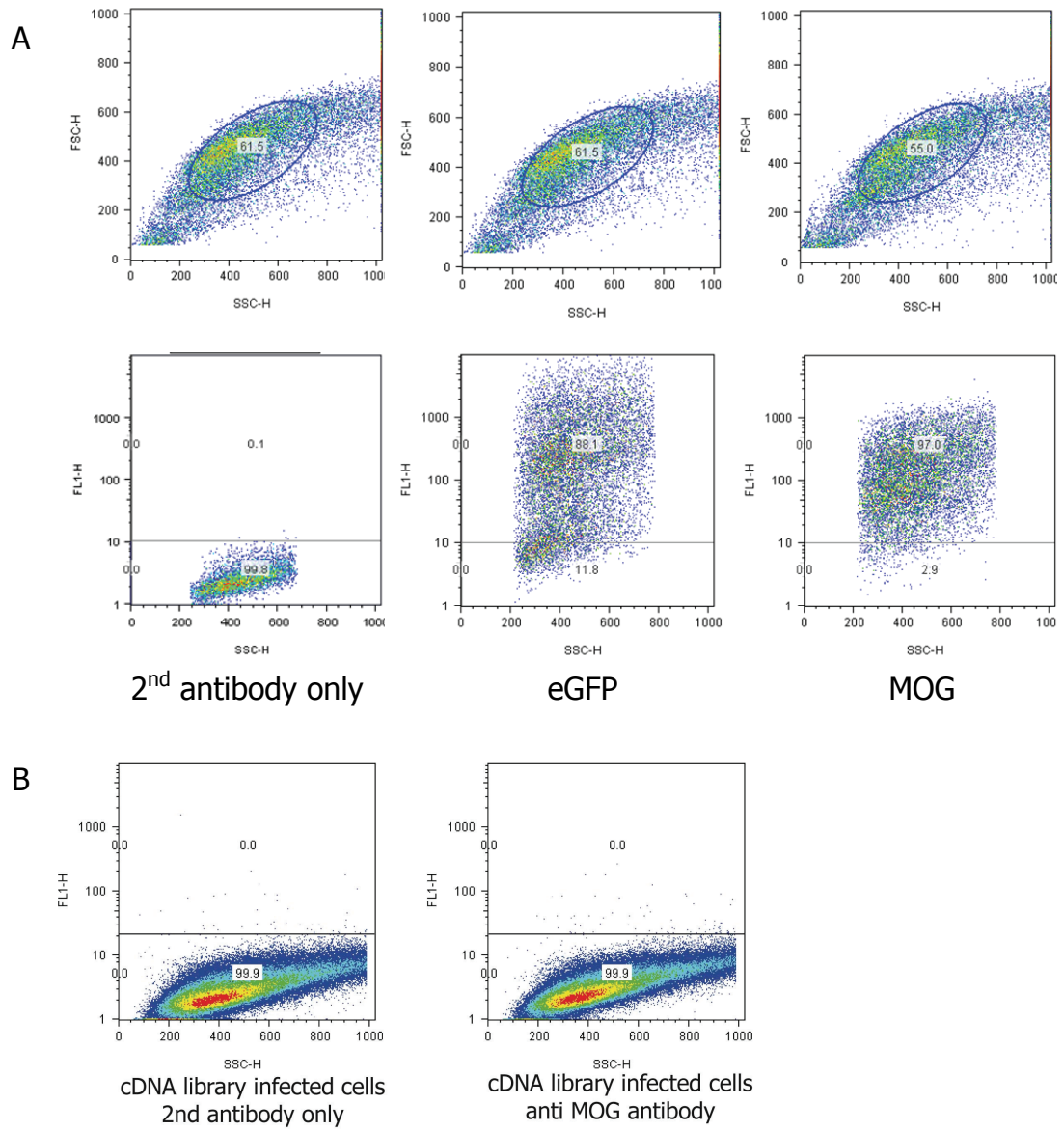


Figure 21. FACS analysis for the verification of retroviral constructs.
A: In this analysis two constructs have been verified for their functionality. eGFP and MOG was recombined into the retroviral expression vector pQCXIP. eGFP expression was visualized by direct FACS analysis, MOG infected cells were stained using specific antibodies.
B: These two FACS panels represent the experiment with the goal to identify MOG expressing mouse cells, which were previously infected by virus containing the human brain derived cDNA library.

4 Discussion

DM, IBM, and PM are distinguishable syndromes in the group of IMs. DM is thought to be primarily an antibody-mediated autoimmune disease, in which antibodies recognizing endothelial antigens activate the complement cascade (Dalakas and Hohlfeld, 2003). This activation could lead to a destruction of intrafascicular capillaries and subsequently to perifascicular myofiber atrophy induced by ischemia. However, the presence of such autoantibodies has never been proven.

IBM and PM are thought to be T-cell mediated. Cytotoxic T cells surround and also invade the muscle fiber, expressing cytotoxic molecules, such as granulysin, perforin, and granzyme A (Goebels et al., 1996; Ikezoe et al., 2006; Orimo S et al., 1994). In IBM, a degenerative process of muscle fiber injury is also present.

However, the immune mechanisms leading to the pathologies in IM are still controversially discussed. Recent publications from Greenberg et. al. (in which new technologies have been applied to study IM) have led to the discovery of new cell types and factors, which seem to be involved in these diseases.

4.1 Inflammatory myopathies

4.1.1 Dermatomyositis

Histological analyses of several cases of dermatomyositis have shown the presence of B- and PCs, which is in line with common concepts, but recently plasmacytoid dendritic cell (pDC) infiltration as well. The presence of these main producers of type I interferons (pDC) induces the expression of IFN-α/β dependent genes like myxovirus resistance A (MxA), STAT-1 and MHC-1 in capillaries and perifascicular fibers (Nagaraju et al., 2005; Gallardo E et al., 2001; Greenberg et al., 2005b). The type I interferon production of pDC might be induced by immune complexes (consisting of antibodies against RNA or DNA and its antigen) which are binding to Fc receptors on the pDC and activate intracellular signaling cascades after internalization via toll like receptor 7 and 9.

PDCs present antigens to T helper or T regulatory cells and can induce the differentiation of B cells into PCs (Boonstra et al., 2003; Nestle et al., 2005; Jego G et al., 2005). One other possibility is that a viral infection triggers IFN-α/β production. However, a viral infection has never been observed in IM (Dalakas, 2006b; Jongen et al., 1993; Leff et al., 1992).

The production of IFN-α/β might have several consequences. Other cell types of the innate immune system, such as myeloid dendritic cells, monocytes, and pDCs, are activated by

interferons. B cells are induced to produce more anti-DNA antibodies. A further hypothesis is that IFN-α/β might contribute to muscle and endothelial cell injury.
This concept suggests a strong interaction between the adaptive and innate immune system as observed in established autoimmune diseases, such as systemic lupus erythomatosus.

However, further questions have to be answered addressing the identity of the target antigens. In this context an endothelial or nucleic acid antigen would indicate an autoimmune process, whereas a viral antigen would neglect autoimmune mechanisms involved in the pathogenesis of dermatomyositis.
The presence of an IFN-α/β gradient with peak concentration in perifascicular (pf) areas and capillaries does not explain why capillaries within the whole fascicle are damaged. The mechanism underlying why pf myofibers upregulate IFN-α/β induced genes and are affected is still unknown (Hohlfeld and Dornmair, 2007).

4.1.2 Inclusion-body myositis and polymyositis

First histological analyses have been done to characterize the T-cell infiltrate for all T cells, cytotoxic and suppressor T–cells and helper T-cells (Arahata and Engel, 1984; Engel and Arahata, 1984; Arahata and Engel, 1986). These studies done in the 1980s have some limitations in view of our current knowledge of T-cell biology. The antibodies used to stain all T cells were specific for the β-chain of CD8 T-cell receptors, and the staining therefore only revealed all $CD8^+$ T-cells. The antibody for $CD4^+$ helper T-cells also stains macrophages and dendritic cells because they express the CD4 receptor as well. These restrictions led to wrong calculations of the number of all cells and helper T-cells.
The analysis of the T-cell receptor repertoire in IBM and PM by spectratyping, laser microdissection and sc-PCR of the TCR transcripts has demonstrated the presence of a clonally restricted populations of T cells in IBM and PM muscle (Hofbauer et al., 2003; Fyhr et al., 1997). Microarray analyses have shown that Ig transcripts are highly expressed in IBM and PM (Greenberg et al., 2005a). Histological analyses for PCs verified their presence in muscle tissue as the main producers of Igs. These sequences show all features which characterize affinity maturation by a GC reaction.
A further recent finding was the identification of myeloid dendritic cells (mDCs), surrounding and invading non-necrotic muscle fibers (Greenberg et al., 2007). mDCs are professional antigen-presenting cells and might, therefore, contribute to the pathogenesis in IBM and PM, by forming immunological synapses with T cells and also B cells.

The question, which trigger initiates the inflammation of the muscle remains unsolved. What are the antigens of the expanded PCs and T cells? Do they target the same antigen by linked recognition? Are cells present in the muscle, which are necessary for an ectopic germinal center reaction in the muscle such as FDCs?

Bradshaw et al. recently described the presence of B- and PCs in IM. They characterized the Ig repertoire by creating a hc Ig cDNA library from isolated RNA from whole tissue sections and showed the presence of clonally expanded B and PCs and Ig sequences with features of a GC reaction.
However, that study has some limitations. First of all, by cloning total RNA (isolated from muscle biopsies) it was impossible for them to distinguish between Ig produced by B cells or by PCs. Furthermore, detection of clonal expansions is difficult due to technical limitations. Expansions are only detectable by cloning Ig cDNA from different areas of the same section. Additionally, it is not possible to determine whether the B cells originate from the tissue or from blood vessels.

The objective of this study was a detailed analysis of the PC repertoire in inflammatory myopathies. Several biopsies from IBM, DM, and PM patients containing PC infiltrates were analysed using two independent approaches: For the analysis of the overall clonal composition of the B- and PCs contained in individual biopsies, we determined the CDR3 length distribution of Ig hc transcripts by CDR3 spectratyping. For the more detailed analysis of the hc and lc Ig sequence of individual tissue-infiltrating PCs, we applied a combination of LM and sc-RT-PCR of microdissected cells.

4.2 Results from IM repertoire analyses

In our study we analyzed the PC repertoire in five biopsy samples of IBM, four of DM, and two of PM. All cases were clearly diagnosed diseases and were verified by a histopathologist. In one analyzed IBM biopsy we observed a prominent PC infiltrate with Ig genes of IgG isotype. After analyzing eighty sequences, we had clear evidence of clonal expansions of at least eight clones. These clones were found in separate experiments, and two of them were verified by CDR3 spectratyping. Except in one clone, all sequences were identical in their nucleotide sequence, containing a moderate to high number of somatic hypermutations, which characterizes affinity maturation. The finding of higher R/S ratios in the CDR regions of expanded clones compared to lower ratios in the CDR regions of non-expanded clones provides further evidence for this phenomenon. Additionally we found strongly expanded PCs in two IBM biopsies containing replacement mutations, but no clonal variation.

Analyses of PCs infiltrating muscle tissue of DM and PM patients indicate a polyclonal PC repertoire with weak clonal expansions, but more cases of clonal variation (7) than that found in IBM (3).

Our data is in line with the results shown in the publication of Bradshaw et al. However, our study verifies the findings of Bradshaw et al. in a more detailed and definite way by analyzing not only Ig expression from tissue, but also the PC infiltrate, in particular. Our approach allows, on the one hand, a detailed analysis of both heavy- and light-chain Ig of single PCs and, on the other hand, provides information of the PC location in the biopsy. It also indicates clonal expansions in a more direct way, in terms of comparing each hc sequence from individually isolated PCs of independent experiments. Furthermore, the amplification of heavy- and light- chain Ig from the same cell allows the recombinant expression of the antibody and the possible identification of the cognate antigen.

The finding of clonal expansions of PCs in IM is in line with the presence of $CD8^+$ T-cell expansions (Hofbauer et al., 2003). This indicates that a targeted immune response is present in IM, involving the cellular and humoral immune compartments.

4.3 Using LM for the analysis of single immune cells

The laser-microdissection technique was developed in the mid-1990s at the National Institute of Health by Emmert-Buck and colleagues. (Emmert-Buck et al., 1996; Simone et al., 1998). It was first used in the isolation of tumor tissue. This technique was further improved over the last decade.

Laser microdissection in cellular immunology of human diseases is a very suitable technique to dissect single cells from complex tissues. Tissues (and especially diseased tissues such as tumors) are complex three-dimensional structures, which contain highly heterogeneous cells concerning phenotype and morphology. In combination with highly specific immunohistochemical staining procedures, a pure cell population can be isolated. In contrast to fluorescence activated cell sorting (FACS) from previously digested tissue, the information of cellular environment of the target cell type is maintained in LM. Furthermore, LM allows the analysis of RNA, DNA or protein for the detection of gene expression with real-time PCR or Western blotting, or the accumulation of cells for the purpose of mRNA microarrays (Curran et al., 2000).

In our study we were interested in the B-cell response in inflammatory myopathies. The necessary techniques (fast and efficient staining, the isolation of small tissue pieces containing the cell of interest and the amplification of Ig hc and lc genes) had to be established.

Since there are RNA-degrading enzymes not only in the tissue itself but also in labware and buffers, all of these methods required preservation of the RNA integrity. For this reason, all chemicals were of highest available purity. Solutions, which were in contact with the tissue or cell lysate, contained a potent RNase inhibitor.

PCs as target cells have the advantage that they contain 10-20% mRNA of total mRNA coding for the Ig genes, making amplification more efficient. Because of the high amplification power of our PCR, we had to avoid the generation of PCR products from contaminating agents (such as previously cloned PCR products). For this reason, we performed the LM, sc RT-PCR and product purification in different rooms. We always compared newly generated products with sequences we already obtained from other biopsies and never observed any cross-contamination.

Some LM samples were comprised by more than one PC, since we could amplify both Ig hc genes of IgG and IgM isotype. The fact that we amplified the same sequence in different independent LM experiments shows the reproducibility of our method. In these experiments, we observed the correct pairing of the hc to the lc, as well. Additionally, we verified three

expanded sequences by a completely different method. We used CDR3 spectratyping to initially detect clonal expansion and identify the corresponding Ig sequence of the expanded clone. Thus, we showed that the generated sequences from LM experiments were no artifacts, instead, the intrinsic products of the infiltrating PCs.

The sequence repertoire analysis, including several hundreds of Ig hc sequences, gives inside into the PC situation being present at one time in the course of the disease. It neither gives information about time-dependent changes, concerning the repertoire of Ig gene transcripts in the early phase of the humoral immune response, nor about the impact of treatment with immunomodulating drugs or possible antigen spreading events. To meet the technical limitations of LM and sc RT-PCR a second, broader approach, namely CDR3 spectratyping, was used to analyze the clonal composition of the entire B- and PC infiltrate.

4.4 Occurrence of expanded plasma cells in IM tissue

We assume that the B-cell precursors of the expanded clones matured and increased their affinity in the muscle tissue. The presence of clonally expanded PCs with features of affinity maturation implies an antigen-driven immune response in the target tissue. The clonal variation, which we observed in some of the analyzed biopsies, further supports affinity maturation by a GC reaction inside or outside the inflamed tissue. By comparing the mutations between similar sequences, a genealogy has been established. It seems very unlikely that the expanded PC clones migrate into the inflamed tissue by chance, since mechanisms in which antigen-specific cells are recruited by chemokines into sites of antigen persistence are not described. This assumption is verified by the fact, that infiltration by PCs is not a general feature of IM even in cases where a strong infiltrate is present but contains no PCs. Additionally, PCs need specific survival factors and attractant molecules to migrate into inflamed sites. However, we cannot completely exclude that PCs are unspecifically recruited to the inflamed muscle by such factors, but this seems unlikely since PC infiltration is not a general feature of IM.

4.5 Do plasma cells play a role in disease pathogenesis?

GC-experienced plasmablasts home to the bone marrow by CXCR4–CXCL12 interaction, where they differentiate into PCs and receive survival signals (Hauser et al., 2002). These signals keep the PCs alive by inducing an unfolded protein response. Otherwise, the cell would undergo apoptosis due to endoplasmic reticulum stress, which is generated by high antibody production (Iwakoshi et al., 2003). At this location, the PCs fulfill the function of maintaining protective antibody serum levels and immunological memory.

Under inflammatory conditions, chemoattractants (addressing PCs) are produced in inflamed tissues induced by IFN-γ. These ligands which are CXCL9-11 bind to CXCR3 on PCs and direct them to the site of inflammation (Muehlinghaus et al., 2005; Baggiolini, 1998; Manz et al., 2002; Hauser et al., 2002).

These antibody-secreting cells are also detected in chronically inflamed tissues (Cassese et al., 2003; Mallison, III et al., 1988). As an example for the presence of autoantibodies in sites of inflammation, is e.g. systemic lupus erythomatosus (SLE) (Hutloff et al., 2004), in which also autoantibodies to intracellular antigens are observed as well in rheumatoid arthritis (RA) (Tsubaki et al., 2005; Schroder et al., 1996). The presence of PCs might have two functions. Either antigen-specific cells are located in the inflamed tissue to produce high

amounts of antibodies which bind to pathogens (beneficial), or the antibodies are directed against autoantigens, which would contribute to a more devastating disease pathogenesis. At these sites, PCs acquire survival factors as well as in the bone marrow, such as CXCL12, IL-6, BAFF and/or APRIL and TNF. These findings however do not explain the occurrence of autoantibodies in these diseases. (Hirano and Kishimoto, 1989; Schneider, 2005; O'Connor et al., 2004; MacLennan and Vinuesa, 2002; Hauser et al., 2002).

We assume, according to comparisons of the expanded clones, that we analyzed the PC Ig repertoire after completing the GC reaction, since such a reaction leads to clonal variation of the B-cell receptor, but as a consequence only the clones with the highest affinity for its antigens survive. However, we cannot exclude an alternative pathway of affinity maturation outside the GC, which has also been demonstrated (William et al., 2002).

Furthermore, a plasmacytoma, which would explain the presence of PCs in inflamed tissues but without any correlation to the immune response in IM, can be excluded according to the clinical history of the patients. It is tempting to speculate, that these antibodies produced by expanded PCs are specific for a muscle autoantigen or for an unknown pathogen.

A recent finding published by Serafini et al. shows a dysregulated Epstein-Barr virus infection in B- and PCs in the brain of MS patients (Serafini et al., 2007).

Epstein-Barr virus (EBV), a human herpes virus, latently infects B-lymphocytes and can also cause infectious mononucleosis. Infected B cells and PCs are found in ectopic lymphoid follicles in the meninges of MS-patients. The authors have shown that EBV-infected B cells are the target of $CD8^+$ cytotoxic T-cells, and that the pathological symptoms in MS might be caused as a secondary effect of the immune response against the EBV-infected cells. We hypothesize that EBV might play a role in IM by infecting B cells, driving them into clonal expansion and further differentiation into memory B-cells and PCs (Kuppers, 2003). The cells, therefore, might undergo an unspecific GC reaction and would not necessarily play a directly disease-related role in the cause of muscle destruction in terms of being specific for a muscle or autoantigen (Brauninger et al., 2003). On the other hand it could possibly be an autoreactive B cells, specific for a muscle autoantigen and escaping tolerance mechanisms due to EBV infection.

4.6 Long-lived plasma cells and immunopathology

The generation of autoantibodies is a general finding in autoimmune diseases. Examples of classical autoimmune diseases are Hashimoto´s thyroiditis (Chiovato et al., 2003), antiphospholipid syndrome (de Groot and Derksen, 2005), myasthenia gravis (Hughes et al., 2004) and bullous skin diseases (Amagai, 1999). These antibodies can be used as a diagnostic marker (Martin and Chan, 2004; Lipsky, 2001; von Muhlen and Tan, 1995).

In SLE, autoantibodies against intracellular antigens are present and form immune complexes, activate complement and bind to Fc receptors (Martin and Chan, 2004). The defective clearance of apoptotic material might contribute to disease severity (Carroll, 2004). These antibodies also show features of somatic hypermutations and class switch recombination which suggests a T-cell-dependent activation (Shlomchik et al., 1990; Brard et al., 1999). The serum concentrations of antibodies are stable for special autoantigens but fluctuate for other autoantigens during the course of the disease. One explanation could be that stable serum concentrations are produced by long-lived PCs and the fluctuating concentrations by short-lived PCs or plasmablasts. This is underlined by the presence of plasmablasts in the blood, with highly mutated Ig genes (Jacobi et al., 2003; Odendahl et al., 2000). The presence of autoantibodies is well-known for IM, especially for DM where 20% of patients have antibodies against nuclear and cytoplasmic antigens, associated with protein synthesis and translational transport (Targoff, 2002).
Autoantibodies are assumed to cause depletion of capillaries in DM, activating complement on endothelial cells. This immune reaction would explain perifascicular myofiber atrophy. Further research, using recombinant antibodies derived from DM tissue, might finally proof the presence of such autoantibodies.

4.7 Identification of target antigens by expression cloning

Since the characterization of the hc and lc Ig gene repertoire indicates the presence of an antigen-driven immune response in IM, the identification of the cognate target antigens would give insight into the factors which trigger the diseases. Therefore, the establishment of a system which would identify such antigens in IMs and also other neurological diseases e.g. MS would potentially contribute to our final goal of investigation.

This system should resemble as much as technically possible the native protein antigens and additionally be highly specific and allow a rapid identification. The most suitable system in our opinion would be a eukaryotic expression system including all posttranslational modifications. Additionally, viral-mediated gene transfer should allow a good control over the number of genome-integrated transgenes per cell and should contribute to a simple identification of the integrated provirus, as well.

In the beginning of our study, much effort has been spent on the construction of retrovirally mediated cDNA libraries from muscle and brain material. Many factors have contributed to the decision to give up this part of the project. Some of them are discussed below.

4.7.1 Limitations of the approach

a) Reduced antigen display

Antibodies usually recognize linear epitopes or conformational epitopes. Both groups of epitopes can be produced using the mammalian-library approach. If the epitope, however, is generated by proteins which build heterodimers or higher order of complex formation, the epitope cannot be mimicked by our system, because only one particular protein is expressed by one infected cell.

b) Lacking normalization

The transcription of genes is dependent on the cell type and directly regulated by promoter activation. The quantity of different mRNAs can vary between five up to thousands of mRNA copies of one gene, so it is very likely, when the antibody is recognizing a low expressed protein, that it is only very little represented in the recombinant library. The cDNA library is further contaminated by highly expressed transcripts, e.g. housekeeping genes of the cell. In a normalized library the transcripts are represented more equally, because highly abundant transcripts are reduced in their quantity.

c) Truncated cDNAs

The quality of the library is dependent on several different factors. One very important criterion is the quality of the RNA which is used for reverse transcription and also the reverse transcription reaction. In both cases, a bad quality or suboptimal reaction conditions might lead to the generation of cDNA fragments, which do not contain the whole coding sequence. The resulting polypeptide chains are not complete or the translation will be out of frame, when an alternative start codon is used.

d) Infection and toxic inserts

In our experiments it was very difficult to measure the viral titer of the library virus. We always transfected and infected the same plasmid containing eGFP in a parallel experiment. This allowed us to get a first hint on the transfection efficacy of the packaging cell line and of the viral titer of the eGFP virus. However, these findings cannot be directly transferred to the library containing virus, because the viral titer is not only determined by the vector backbone, but also by the cDNA sequence length. Because of this, the viral titer should be calculated directly by selecting library infected cells with puromycin. This procedure is very time-consuming and often led to mistakes. As an alternative, we recombined the library into the same expression vector, which instead of a puromycin selection marker contains an IRES eGFP. We infected cells with such virus, but all supernatants were of low titer.

4.8 Future research and application

Our experimental approach allows the resurrection of the whole antibody molecule with the same antigen specificity as produced by the patient immune system, as we have obtained hc and lc Ig from one individual PC. With the antibodies from expanded clones in hands, it would be the final goal to identify the target (auto)antigen. This might help to explain the perifascicular myofiberatrophy in dermatomyositis and muscle fiber destruction in IBM and PM.

If the target antigen is of pathophysiological significance, therapeutic approaches should target the B- and PCs. One monoclonal anti-CD20 antibody (Rituximab®) is already undergoing a clinical trial. However, it would only target $CD20^+$ B cells but not PCs (Clinicaltrial.gov). Other approaches should target the pDC and mDC present in DM and IBM, to prevent secretion of IFN-α/β as well as antigen presentation. Since $CD8^+$ T-cells are also clonally expanded and attack and invade muscle fibers, they might be a therapeutic target in IM (Clinicaltrial.gov).

4.9 Conclusion

Our study gives insight into the role PCs may play in the immunopathogenesis of IM. Our approach provides a detailed analysis of the PC repertoire by two independent methods that are CDR3 spectratyping and sc RT-PCR. According to strong clonal expansions of PCs with highly mutated Ig genes present most prominently in IBM biopsies, we have strong evidence for an antigen-driven immune response including affinity maturation. LM of single PCs provides both information of the location of cells and the sequence of heavy and light chain Ig genes. This approach allows further identification of the corresponding antigens by the resurrection of the original antibody specificity. Further research might reveal the target antigens and provides information of the trigger of the diseases.

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6 Supplementary tables

No	Date of LM	Cell	V name	Identity (%)	D name	Identity (%)	J name	Identity (%)	CDR3 sequence heavy chain	Isotype	CDR3 length
DM-1 (14/06)											
1	070411	21	V3-15*01	96.45	D2-8*01	n.d.	J5*02	80.43	C A T Q Y K T L	IgG	6
2	070411	5	V3-15*01	94.81	D6-13*01	100.00	J5*02	75..61	C T G G K S G H W	IgM	7
3	070321	4	V3-15*01	94.93	D4-17*01	71.42	J4*02	89.58	C A T E D Y G D L G Y W id	IgG	10
4	070411	15	V3-15*01	94.74	D4-17*01	71.42	J4*02	89.58	C A T E D Y G D L G Y W id	IgG	10
5	070411	22	V3-7*02	98.89	D1-14*01	62.50	J4*02	79.16	C A R E I K R G G D D W	IgM	10
6	070321	1	V1-69*01	94.32	D3-16*01	80.00	J4*02	85.41	C A R D L G Q L S L Y F W	IgG	11
7	070411	13	V3-74*02	93.33	D3-16*01	66.66	J4*02	97.91	C A R D R R G Y Y F D Y W	IgM	11
8	070212	4	V1-2*02	94.12	D3-9*01	82.35	J4*02	89.58	C A R A D I L T G N N L D F W	IgM	13
9	070212	24	V3-23*04	97.84	D4-17*01	53.33	J4*02	85.41	C T K D R P V T K R L F D Y W di	IgM	13
10	070212	26	V3-23*01	93.33	D1-1*01	66.66	J4*02	87.50	C T K D R P M T K R L F D N W di	IgM	13
11	070321	11	V3-23*04	94.81	D4-17*01	53.33	J4*02	87.50	C T K D R P V T K R L F D Y W di	IgM	13
12	070411	26	V4-39*01	97.29	D6-19*01	66.66	J4*02	85.41	C A R P R R G N G S G F D Y W di	IgG	13
13	070411	32	V4-39*01	97.46	D6-19*01	66.66	J5*01	82.35	C A R P R R G N S S G L D Y W di	IgG	13
14	070411	8	V3-64*05	86.38	D5-24*01	71.42	J5*01	74.50	C M I V G R D S N S L G Q A S W	IgG	14
15	070411	16	V1-3*01	98.91	D2-15*01	85.00	J4*02	83.33	C A R G I C S G G S C P F A Y W	IgM	14
16	070411	31	V3-30*01	97.19	D3-3*01	80.00	J5*02	80.39	C A R D R M S Y G D H G P G N W	IgM	14
17	070212	2	V3-23*01	97.80	D6-13*01	58.82	J4*02	85.50	C A K D R R G S S K G L F F D Y W	IgM	15
18	070212	7	V3-23*01	96.34	D3-22*01	78.94	J4*02	77.08	C A T Q K R Y F D S S G Y L G D W	IgM	15
19	070212	21	V1-69*06	93.05	D1-26*01	77.77	J5*02	88.23	C V F S R G V G A T T Y W F D P W	IgM	15
20	070321	8	V3-23*04	98.90	D4-11*01	56.25	J4*02	83.33	C A K D R I E P T T Y R G H D Y W	IgM	15
21	070411	6	V3-33*03	88.15	D2-8*02	64.28	J5*02	86.27	C A R D G G N I I L V G W F D A W	IgG	15
22	070411	9	V4-39*01	87.46	D5-24*01	58.82	J3*01	86.00	C A R H S L Y R L P Q S G A F D V W	IgG	16
23	070411	19	V3-20*01	98.89	D6-19*01	85.71	J3*02	96.00	C V R G G Y S S G W R R N A F D I W	IgG	16
24	070411	20o	V1-3*01	89.13	D3-9*01	72.72	J6*03	87.09	C V R V Q Y F S D L G Y H Y M D V W	IgG	16
25	070212	1	V3-23*01	98.53	D2-2*01	76.92	J4*02	85.41	C A K G A A I V L V P A A T P F D Y W	IgM	17
26	070321	2	V3-73*01	100.00	D4-17*01	68.75	J6*03	88.70	C T R P Y G D H G G G Y Y Y Y M D V W	IgG	17
27	070411	7	V7-4-1*02	95.93	D5-18*01	55.00	J5*02	94.11	C A R G R R I D V S I Q K A W F D P W	IgG	17
28	070411	2	V4-39*01	96.18	D3-3*01	75.00	J2*01	71.69	C A R R E R L L E W S S T W Y F D L W	IgM	17
29	070212	10	V3-53*01	98.17	D3-10*01	72.41	J4*02	95.83	C A R E R R T M V Q G V N L Y Y F D Y W	IgM	18

30	070212	14	V3-64*05	97.04	D6-19*01	65.00	J3*02	86.00	C A K V S P R R T T V A V T G A F D I W	IgM	18
31	070321	14	V3-53*01	94.01	D6-13*01	92.30	J6*03	82.25	C A R D R G I A A S G Q S Y Y Y M D V W	IgM	18
32	070411	18	V3-53*01	96.74	D3-10*01	69.23	J6*03	80.64	C A R D R S A Y G S G S H Y Y Y M D A W	IgM	18
33	070411	20	V3-53*01	96.74	D6-19*01	100.00	J6*03	87.09	C A R V K G I A V A G Q N Y Y Y M D V W	IgM	18
34	070411	29	V3-53*01	95.13	D3-10*01	57.69	J5*02	86.27	C A R D R R V L F Q G V T S R W F D P W	IgM	18
35	070411	32	V3-53*01	97.46	D3-10*01	64.28	J6*03	75.80	C A R D R A T M F Q G L T F R Y M D V W	IgM	18
36	070321	11	V1-8*01	88.89	D2-2*01	56.66	J3*01	92.00	C A T R P S K Y Q M L S P T S D A F D L W	IgG	19
37	070411	19	V3-23*04	93.68	D3-3*01	51.61	J3*02	86.00	C A K D R V R F L E W A T T X D A V D I W di	IgM	19
38	070411	27	V3-23*04	96.01	D3-3*01	61.29	J3*01	90.00	C A K D R L R F L E W A T K I D A F D V W di	IgM	19

No	Date of LM	Cell	V name	Identity (%)	D name	Identity (%)	J name	Identity (%)	CDR3 sequence heavy chain	Isotype	CDR3 length
DM-2 (354/03)											
1	070105	28	V3-30*03	92.11	D6-13*01	72.73	J4*02	83.33	C A K A P A D P V G S W	IgG	10
2	070129	2	V3-33*01	85.88	D1-1*01	83.33	J4*02	82.93	C A R D F R T G M T D Y W	IgG	11
3	070129	35	V4-59*01	87.04	D5-24*01	80.00	J2*01	70.59	C A R E A I R R F F D L W	IgM	11
6	070105	13u	V1-3*01	95.12	D5-18*01	63.64	J4*02	79.17	C A R V Y G G L P F D Y W	IgM	11
4	070129	29	V3-73*01	95.79	D5-12*01	76.92	J4*02	87.50	C T S G Y D Q T Y F D Y W	IgM	11
5	061215	21	V1-69*01	94.04	D3-16*01	61.53	J4*02	72.91	C A R A D Y E S G L V E D W	IgM	12
7	070129	26	V1-69*01	92.01	D4-17*01	100.00	J4*02	85.42	C T R G D Y A E G L D D Y W	IgM	12
8	070129	9	V4-b*01	92.91	D5-12*01	75.00	J5*02	90.91	C V R S R D Y L S W F V P W	IgG	12
9	061215	14	V3-48*03	92.67	D4-17*01	81.81	J2*01	96.22	C A R E A Y G E N W Y F D L W	IgG	13
10	070105	8	V1-69*01	90.76	D6-6*01	70.59	J4*02	83.78	C T R G M E Q L A S Y I G Y W	IgM	13
11	070105	18	V3-74*02	93.33	D6-6*01	53.85	J5*02	80.39	C A R G S R P P S C P V D P W	IgM	13
12	070105	24	V3-73*01	89.55	D1-1*01	60.00	J3*02	72.00	C L I V T D T F L D A F H I W	IgM	13
13	070129	27	V4-30-4*01	91.37	D5-12*01	70.00	J4*02	90.24	C A R R L Q W L T D G F D Y W	IgG	13
14	070105	39	V3-23*04	89.82	D1-1*01	72.73	J4*02	82.50	C A K D L A G L R S G F D F W di	IgM	13
15	070129	15	V3-23*04	91.40	D3-9*01	66.67	J4*02	83.33	C A K D L A G L R S G F D F W di	IgM	13
16	070105	34	V1-46*01	96.49	D6-19*01	75.00	J5*02	96.08	C A R E I E Q W V K N W F D L W	IgG	14
17	070105	6	V3-30*03	96.53	D5-12*01	52.63	J4*02	77.08	C A K D R S Y W P L S H F D F W	IgM	14
18	070105	17	V3-23*04	89.93	D4-17*01	66.67	J4*02	87.50	C A K D T T V T G R H Y F D N W	IgM	14
19	070105	33	V3-74*02	91.85	D2-15*01	73.68	J4*02	84.38	C A R P G G C S G G S C F D Y W	IgM	14
20	070129	12	V5-a*01	94.74	D6-19*01	61.90	J4*02	87.50	C A R R S C S G S S C F F D Y W	IgM	14
21	061215	19	V3-64*05	92.22	D6-19*01	62.50	J1*01	75.00	C V T G P R Q W L V P H F H H W id	IgM	14
22	070129	33	V3-64*05	83.33	D6-19*01	62.50	J1*01	75,00	C V T G P R Q W L V P H F H H W id	IgM	14
23	061215	17	V4-4*07	87.41	D5-12*01	60.00	J4*02	89.58	C A R G I K R G R L Y Y F D F W di	IgG	14
24	070129	8	V4-4*07	87.32	D5-12*01	60.00	J4*02	90.24	C A R G I K R G R L Y Y F D F W di	IgG	14
25	061215	18	V3-15*01	90.78	D3-3*01	68.42	J5*02	76.47	C T T D L V F G V P M G W F D P W id	IgM	15
26	070105	12	V3-15*01	91.49	D3-3*01	68.42	J5*02	76,47	C T T D L V F G V P M G W F D P W id	IgM	15
27	070105	15	V4-59*02	92.67	D3-3*01	78.26	J4*02	81.25	C A R S A T A F G V V I H F D Y W	IgM	15
28	070129	30	V3-64*05	94.07	D2-21*01	55.56	J4*02	91.67	C V K E R V N G P S R Y Y F D C W	IgM	15
29	070129	3	V3-20*01	95.24	D5-24*01	70.00	J6*03	87.10	C A R P E F R K D Y H Y Y M D V W	IgG	15
30	070105	5	V3-21*01	95.56	D6-13*01	58.82	J5*02	92.16	C V R E L E H L T L G G D W F D P W	IgG	16
31	070105	17	V1-46*01	85.66	D3-10*01	56.52	J4*02	85.42	C V R G G S T L D E T D S F G G Y W	IgG	16

32	070105	27	V6-1*01	91.67	D6-19*01	84.21	J3*02	98.00	C T R T G V D S S G W F D A F D I W	IgG	16
33	070105	35	V3-23*04	89.61	D2-21*02	75.00	J1*01	80.77	C V R D L R R G S C G G D C P P G W	IgG	16
34	061215	16	V3-23*04	91.23	D3-22*01	89.47	J5*02	94.11	C A K G F T Y Y Y D S S E D W F D P W	IgG	17
35	070105	25	V3-49*03	95.79	D6-13*01	100.00	J6*02	80.65	C T R D S S S W S S K G L K G M D V W	IgG	17
36	070105	7	V3-23*04	93.90	D5-24*01	75.00	J4*02	87.50	C A K P S R Y G S G E K P Y Y F D Y W	IgM	17
37	070105	29	V1-18*01	92.36	D2-15*01	55.56	J4*02	81.58	C A R D S S W E V P A K S L L V D F W	IgM	17
38	070105	37	V3-23*04	97.78	D5-12*01	69.57	J4*02	90.00	C A K D N L D F L A T I P P N F D Y W	IgM	17
39	061215	20	V1-69*06	88.77	D3-10*01	61.53	J3*01	74.00	C A V E S N Y I N S G S P L L A L D V W	IgM	18
40	070105	43	V1-69*06	94.04	D3-10*01	69.23	J3*01	70.59	C G L E S N Y F G S G I P L L A L D V W	IgM	18
41	070105	36	V3-53*01	88.65	D3-3*01	65.38	J4*02	85.42	C T R E R R L S V S G V T N A L F D R W	IgM	18
42	070129	10	V3-7*02	96.34	D6-6*01	88.89	J4*02	83.33	C A R D Q P R I A A R P P R P Y F D N W	IgM	18
43	070129	16	V1-69*06	92.47	D3-10*01	65.38	J3*01	76.00	C A L E S D Y F G S R S P L L A L D V W	IgM	18
44	070129	18	V3-74*02	91.47	D4-23*01	57.89	J4*02	96.88	C A R S A A L R G Q L V P T Y Y F D H W	IgM	18
45	070129	32	V4-39*01	97.13	D6-13*01	76.19	J2*01	77.36	C A S H R G M A A A D I V Y W Y F D L W	IgM	18
46	070129	5	V3-30*05	91.67	D6-13*01	90.48	J2*01	84.91	C A R E A G H S S S W S P G R Y F D L W	IgG	18
47	070129	24	V4-61*02	95.83	D3-3*01	86.21	J4*02	95.12	C A R T G I T L F G V V I E A Y F D Y W	IgG	18
48	070105	2	V3-74*02	94.74	D3-3*01	85.19	J2*01	95.12	C A R D R I P I F G V V I P L W Y F D L W	IgM	19
49	070105	42	V2-5*10	98.26	D3-10*01	88.46	J5*02	90.20	C T Q R P P I T M I Q G V I G N W F D P W	IgM	19
50	070105	19	V3-23*01	87.37	D2-15*01	81.25	J6*03	91.94	C A R S L R D C S G G A C Y Y Y Y M D V W	IgG	19
51	070129	11	V4-31*02	87.02	D3-3*01	74.19	J4*02	83.33	C A R G G V T V F G V V I N P F H F D T W	IgM	19
52	070129	31	V3-23*04	92.63	D3-10*02	66.67	J4*02	95.83	C S K A P T I V F G D L L F I N Y F D Y W	IgM	19
53	061215	15	V1-2*02	90.88	D3-3*01	75.86	J3*02	86.00	C A R G N P T S G F W T A Y Y P G T F D I W	IgG	20
54	070105	14	V4-30-2*01	94.79	D2-2*01	77.42	J3*02	94.87	C A R A P D I V V V P V A I M G G A F D I W	IgM	20
55	070129	6	V1-69*06	92.96	D3-22*01	56.00	J4*02	87.50	C A R D L N R R F P F D R A G W G P F D F W	IgG	20
56	070129	19	V4-4*07	95.13	D6-19*01	84.21	J6*02	84.91	C A R G G G R I T V A G K L L S H Y G M D V W	IgM	21
57	070129	14	V1-69*06	91.29	D2-21*02	80.00	J6*02	88.71	C S R D S Y C G A D C H L D Y F Y Y G M D V W	IgG	21
58	061215	22	V4-61*01	91.67	D3-10*01	65.00	J6*03	82.25	C A G V R V E A T L V Q G V V L Y Y Y Y M D V W	IgM	22
59	070105	36*	V3-23*04	94.98	D3-10*01	76.92	J4*02	87.50	C A K T M V R G V I G G S G E R L T N Y F D H W di	IgM	22
60	070105	13	V3-23*04	98.96	D3-10*01	76.92	J4*02	87.50	C A K T M V R G V I G G S G E R L T N Y F D C W di	IgM	22
61	070129	34	V3-23*04	97.49	D3-10*01	76.92	J4*02	93.75	C A K T M V R G V I G G S G E R L T H Y F D Y W di	IgM	22
62	070105	40	V1-69*06	91.49	D3-22*01	61.29	J3*02	86.00	C A T Y T R R R F R N F D R G G Y S E A F P F D I W	IgG	26

No		Cell	V name	Identity (%)	D name	Identity (%)	J name	Identity (%)	CDR3 sequence heavy chain	Isotype	CDR3 length
DM-3 (289/95)											
1	070319	9	V4-34*01	95.08	D3-16*01	83.33	J5*02	70.58	C A R L G V T A I S L W id	IgM	10
2	070319	28	V4-34*01	95.79	D3-16*01	83.33	J5*02	69.77	C A R L G V T A I S L W id	IgM	10
3	070319	17	V5-51*01	96.67	D3-10*01	100.00	J2*01	96.22	C A R H P N W Y F D L W	IgG	10
4	070314	3	V3-11*03	91.38	D5-18*01	70.00	J3*02	90.00	C A R A M A S D A F D M W	IgG	11
5	070319	24	V1-18*01	98.52	D4-11*01	57.14	J5*02	94.11	C A R T L S N R W F D P W	IgG	11
6	070319	25	V1-3*01	90.68	D5-12*01	69.23	J4*02	79.16	C T R G M L A Q L F E F W	IgG	11
7	070314	21	V3-30*02	88.93	D2-21*01	62.50	J4*02	91.66	C A K D G G N G W Y Y F D Y W	IgG	13
8	070319	22	V1-69*01	90.48	D3-22*01	58.82	J4*03	83.33	C A R A P M K V G G Y Y G A W	IgG	13
9	070319	35	V4-59*01	87.12	D3-10*01	100.00	J6*02	75.80	C A R S E A A T P Y G L D V W	IgG	13
10	070314	10	V1-18*01	83.33	D2-2*01	57.14	J5*02	84.31	C V T S R G A W P R D W F E A W	IgG	14
11	070314	19	V3-64*05	96.13	D2-15*01	66.67	J3*01	88.37	C V R L Y V V V G V G A F D L W	IgM	14
12	070319	10	V4-59*01	87.41	D3-3*02	66.66	J6*02	72.58	C A R Q P F W S G Y L Y G L D V W	IgG	15
13	070319	12	V5-51*01	95.57	D5-24*01	100.00	J4*02	93.75	C A R H R S R R D G Y N S F D Y W	IgG	15
14	070319	31	V3-23*01	90.84	D2-15*01	77.27	J4*02	87.50	C A K G K F C S G G N C L S D Y W	IgG	15
15	070319	13	V3-33*01	98.90	D3-22*01	70.00	J2*01	88.67	C A R D S S S D N S C P G Y F D V W	IgG	16
16	070314	5	V4-39*01	99.28	D6-19*01	86.66	J3*02	96.00	C A R Q A V A G I Y L S P G A F D I W	IgG	17
17	070314	6	V4-4*02	93.40	D3-10*02	52.38	J4*02	93.75	C A R G S T Q L V W S S I I Y F D Y W	IgG	17
18	070314	9	V3-21*01	86.74	D3-22*01	67.74	J4*02	79.16	C A R S Y Y H D P S G Y R R T P F E H W	IgG	18
19	070314	17	V4-b*02	96.52	D3-22*01	100.00	J3*02	90.00	C A I S T Y Y Y D S S G Y L G A F D I W id	IgM	18
20	070319	12	V4-b*02	95.57	D3-22*01	100.00	J3*02	90.00	C A I S T Y Y Y D S S G Y L G A F D I W id	IgM	18
21	070319	21	V4-39*06	95.13	D3-16*02	60.00	J5*02	98.03	C A R D V L V I L P V G I K N W F D P W	IgG	18
22	070319	6	V1-69*01	98.52	D3-22*01	96.29	J1*01	94.23	C A T C T Y Y Y D S S G Y Y L E Y F Q H W	IgG	19
23	070319	9	V3-7*01	92.22	D1-26*01	55.55	J6*02	90.32	C A R D Y R V G G P W D Y D Y Y G L D V W	IgG	19
24	070319	27	V5-a*01	95.56	D2-2*01	56.66	J5*02	86.27	C A R P S C S S N T C P T C S C K F Y P W	IgG	19
25	070314	2	V5-51*01	91.11	D3-16*01	68.42	J6*02	88.70	C A R Q G G Y P N S R M V D Y Y Y G M D V W	IgM	20
26	070314	13	V1-3*01	90.74	D6-13*01	66.66	J6*02	87.09	C V R G A V H S G S W E W G P K N R Y F Y A M D V W	IgG	24
27	070314	4	V3-23*01	90.00	D2-2*01	74.19	J4*02	91.66	C A K G R G S C S G T N C W T I F A V V R Y F D S W	IgG	24

No	Date of LM	Cell	V name	Identity (%)	D name	Identity (%)	J name	Identity (%)	CDR3 sequence heavy chain	Isotype	CDR3 length
DM-4 (354/98)											
1	070328	12	V3-7*01	89.61	D5-5*01	100.0	J3*02	84,00	C A R D L R V F D M W	IgG	9
2	070328	16	V1-3*01	89.12	D6-19*01	75.00	J4*02	85.41	C A R G D R S S G F D S W	IgG	11
3	070328	18	V1-2*02	97.49	D2-2*01	55.55	J4*02	87.50	C A R S Q S Q L A V D Y W	IgG	11
4	070404	35	V1-18*01	90.74	D1-26*01	80.00	J4*02	85.41	C A R D G R D D G E D Y W	IgG	11
5	070328	20	V1-46*01	93.48	D3-16*02	53.33	J4*02	85.41	C A R Q A V G V I P Y D Y W	IgG	12
6	070404	1	V3-30*03	70.83	D6-6*01	53.33	J4*03	77.08	C A R A H N I P S P P D A W	IgG	12
7	070328	10	V1-2*02	83.33	D2-8*01	63.63	J3*01	90.00	C A L T A G F Y R D A F D F W	IgG	13
8	070404	9	V3-23*01	82.20	D3-3*01	64.28	J3*02	84.00	C A K D L W R H F N V F D S W	IgG	13
9	070328	14	V4-34*01	64.88	D2-15*01	63.16	J4*02	87.80	C G E E L H G D G G A G F D Y W	IgG	14
10	070328	2	V4-34*08	96.21	D6-13*01	80.00	J4*02	89.58	C A M K S G R R S S W S L D Y W	IgG	14
11	070328	3	V1-2*02	90.06	D5-5*01	66.66	J3*02	92.00	C A R E G G G G N R D G L D M W	IgG	14
12	070404	18	V3-30*09	90.37	D5-12*01	78.57	J4*02	91.66	C A R G F R G Y S G H D L D Y W	IgG	14
13	070404	22	V3-30*03	84.44	D2-2*01	64.28	J4*02	81.25	C A R A R S F A V S S P P D A W	IgG	14
14	070404	23	V3-30*03	88.26	D3-22*01	61.53	J4*02	70.83	C V Q S D S S G F Y L L G G Y W id	IgG	14
15	070404	25	V3-30*03	88.26	D3-22*01	61.53	J4*02	70.83	C V Q S D S S G F Y L L G G Y W id	IgG	14
16	070404	30	V1-2*02	88.04	D7-27*01	85.71	J4*02	83.33	C A R A G Q Q G I H P L I D Y W	IgG	14
17	070328	21	V3-30*03	94.57	D1-26*01	54.55	J4*02	83.33	C A R D R L S R H K V P T I G Y W	IgG	15
18	070328	23	V4-34*01	86.52	D3-9*01	62.96	J4*02	83.33	C A H T T V L T T S Y Q A W E F W	IgG	15
19	070328	27	V3-7*02	74.00	D4-23*01	68.42	J4*02	87.50	C A R D G V G G Y S P R D F D S W	IgG	15
20	070328	1	V2-70*01	85.72	D3-10*01	72.72	J4*02	87.50	C A R T P F Y F G M G S Y F F D Y W	IgG	16
21	070404	24	V4-61*01	92.96	D3-9*01	85.71	J5*02	82.35	C A R A V L R D F D W L L G V D P W	IgG	16
22	070404	29	V3-7*01	95.56	D4-23*01	70.00	J6*02	85.48	C A R V E T P P V P S Y Y G M D V W	IgG	16
23	070328	11	V3-11*01	91.67	D3-22*01	100.00	J4*02	89.58	C A R G G L Y Y Y D S S G V P F D Y W	IgG	17
24	070404	6	V4-59*01	93.94	D2-8*02	55.55	J4*02	75.00	C A R S H P K A V F D W S A C L D S W	IgG	17
25	070404	19*	V1-46*01	96.77	D3-10*02	61.53	J6*02	88.70	C A R D K C E T Q H Y Y Y Y A L D V W	IgG	17
26	070404	32	V4-61*08	83.33	D4-17*01	75.00	J6*02	82.25	C A R V H D Y G A Y A G D Y K Y V M D V W	IgG	19
27	070404	3	V3-30*03	95.93	D6-13*01	68.75	J6*02	85.48	C A K D S S K V V A A V N F W Y Y G M D V W id	IgG	20
28	070404	4	V3-30*03	95.93	D6-13*01	68.75	J6*02	85.48	C A K D S S K V V A A V N F W Y Y G M D V W id	IgG	20
29	070404	31	V1-46*01	91.67	D3-10*01	66.66	J6*02	80.64	C A R D G T H L L G V G E L Y S N G M D V W	IgG	20
30	070328	17	V3-15*01	88.28	D2-8*01	61.53	J3*01	88.00	C T T G P A S V G S C D T D N C P D A F N L W	IgG	21
31	070328	30	V1-2*02	89.26	D3-9*01	77.41	J5*02	78.43	C A R E S R S N Y N I M T A Y Y K D P A E D H W	IgG	22

32	070404	15	V1-2*02	90.53	D6-13*01	61.90	J5*02	94.11	C A R R G R R S I T I G G S A P P D G W F D P W id	IgG	22
33	070404	16	V1-2*02	90.53	D6-13*01	61.90	J5*02	94.11	C A R R G R R S I T I G G S A P P D G W F D P W id	IgG	22
34	070328	4	V1-69*06	90.37	D3-9*01	58.06	J4*02	91.66	C A K V V P A R V L R H F D S A P N L L Y F D I W	IgG	23
35	070328	7	V1-69*06	88.77	D3-3*01	65.38	J6*02	85.48	C A G R Q A S G I S V F G V G P S S Y F H Y P M D V W	IgG	25
36	070404	7	V1-f*01	97.04	D2-2*01	69.56	J6*02	82.25	C A T S T H G P K K P L P Q R R G I V I I P A A N H C F S G V D V W	IgG	32

No	Date of LM	Cell	V name	Identity (%)	D name	Identity (%)	J name	Identity (%)	CDR3 sequence heavy chain	Isotype	CDR3 length
IBM-1 (451/96)											
1	061213	27	V3-11*01	93.18	D5-5*01	90.00	J4*02	85.41	C A K G Y Q Y A H Y W	IgG	9
2	070510	14	V3-23*01	92.80	D4-17*01	60,66	J4*02	89.58	C A Q G D Y G P D Y W	IgG	9
3	070521	37	V3-23*01	88.64	D2-21*02	80.00	J4*02	87.80	C A K G D Y G P D C W	IgG	9
4	061206	15	V3-30*09	88.89	D4-4*01	100.00	J4*02	81.25	C V A D Y R S L G D Y W	IgG	10
5	061221	27	V4-30-4*01	95.29	D6-6*01	71.42	J3*02	100.00	C A R I R D D A F D I W	IgG	10
6	061206	17	V1-18*1	90.94	D6-19*01	83.33	J4*02	83.33	C A R Y T S A C P S C F W	IgG	11
7	061206	19	V3-15*01	94.93	D3-22*01	71.42	J2*01	92.45	C T S Y E S S G W Y F D L W	IgG	12
8	070116	16	V4-61*01	89.24	D4-23*01	n.d.	J4*02	83.33	C A R D D Y A T N T V V D W	IgG	12
9	061206	32	V3-15*07	92.91	D3-3*01	75.00	J4*02	83.33	C S T E R T F G V I L I R D W	IgG	13
10	061213	37	V1-f*01	94.20	D6-6*01	58.33	J4*02	93.75	C A T E V G R S R S S F D Y W	IgG	13
11	061221	28	V5-51*01	96.74	D1-7*01	100.00	J6*02	72.58	C A R Q L E L R R R G M D L W	IgG	13
12	070116	41	V1-f*01	85,42	D1-1*01	87.50	J5*02	80.39	C G T G T N N W H G G I A H W	IgG	13
13	061221	12	V6-1*01	96.77	D6-19*01	75.00	J6*02	80.64	C A R E H W L G H Y Y G M A V W	IgG	14
14	070116	17	V1-3*01	93,19	D3-10*01	100.00	J4*02	93.75	C A S Y Y G S G S Y W F F D Y W	IgG	14
15	061206	13	V1-18*01	88.51	D1-14*01	60.00	J4*02	93.75	C A R G K R G A R D N Y F D Y W id	IgG	14
16	061213	23	V1-18*01	88.51	D1-14*01	60.00	J4*02	93.75	C A R G K R G A R D N Y F D Y W id	IgG	14
17	070510	35	V1-18*01	88,51	D1-14*01	60.00	J4*02	93.75	C A R G K R G A R D N Y F D Y W id	IgG	14
18	061221	15	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
19	061221	19	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
20	061221	20	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
21	061221	22	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
22	061221	23	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
23	061221	29	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
24	061221	31	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
25	070116	27	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
26	070116	28	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
27	070116	30	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
28	070116	40	V3-9*01	97.78	D4-17*01	90.00	J4*02	87.50	C V Q D T V T N L N Y S F A X W id	IgG	14
29	070116	31	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
30	070510	2	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
31	070510	34	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14
32	070510	37	V3-9*01	97.78	D4-17*01	90.00	J4*02	91.66	C V Q D T V T N L N Y S F A S W id	IgG	14

33	070116	18	V4-59*01	92.18	D3-22*01	77.27	J4*02	92.68	C A R T S Y H E S I G Y Y F D Y W	IgG	15
34	070510	7	V3-23*04	93.94	D3-22*01	68.18	J4*02	93.75	C A A E R A Y G S S G Y A F D Y W	IgG	15
35	061221	5	V3-33*02	n.d .	D1-26*01	66.66	J3*02	92.00	C A R N S H G G R S S D A F D I R id	IgG	15
36	070116	33	V3-33*02	69.23	D1-26*01	66.66	J3*02	92.00	C A R N S H G G R S S D A F D I R id	IgG	15
37	070116	21	V3-33*01	87.64	D1-26*01	66.66	J3*02	92.00	C A R N S H G G R S S D A F D I R id	IgG	15
38	061206	30	V4-30-4*01	86.31	D4-17*01	81.25	J5*01	86.27	C A R H L Y D Y G D F A K W L D S W	IgG	16
39	070116	14	V3-13*01	94.01	D6-25*01	82.35	J2*01	88.67	C A R G R G I G A A E T R Y L D L W	IgG	16
40	070510	30	V5-51*01	94.07	D1-26*01	n.d.	J6*02	88.70	C A R F L L G D T Y Y Y Y G M D V W	IgG	16
41	061221	4	V3-74*01	n.d.	D2-2*01	n.d.	J4*02	91.66	C A R R G R L D S T S R F Y F D F W id	IgG	16
42	061221	11	V3-74*02	87.68	D2-2*01	n.d.	J4*02	91.66	C A R R G R L D S T S R F Y F D F W id	IgG	16
43	070116	3	V3-74*02	87.68	D2-2*01	n.d.	J4*02	91.66	C A R R G R L D S T S R F Y F D F W id	IgG	16
44	070116	23	V3-74*02	87.68	D2-2*01	n.d.	J4*02	91.66	C A R R G R L D S T S R F Y F D F W id	IgG	16
45	061221	17	V3-23*04	86.08	D3-16*01	66.66	J4*02	89.58	C A K C G P F G G V M V I P F D Y W id	IgG	16
46	061221	33	V3-23*04	91.67	D3-16*01	66.66	J4*02	89.58	C A K C G P F G G V M V I P F D Y W id	IgG	16
47	070521	27	V3-23*01	88.00	D3-16*01	66.66	J4*02	89.58	C A K C G P F G G V M V I P F D Y W id	IgG	16
48	070510	13	V3-23*04	93.48	D3-16*01	66.66	J4*02	89.58	C A K C G P F G G V M V M P F D Y W id	IgG	16
49	070510	20	V3-33*01	94.12	D2-2*03	68.00	J4*02	82.93	C V R D G Y C T S A N C Y F F Q H W id	IgG	16
50	070510	48	V3-33*01	94.12	D2-2*03	68.00	J4*02	82.93	C V R D G Y C T S A N C Y F F Q H W id	IgG	16
51	070521	38	V3-33*01	94.12	D2-2*03	68.00	J4*02	82.93	C V R D G Y C T S A N C Y F F Q H W id	IgG	16
52	070521	39	V3-33*01	94.12	D2-2*03	68.00	J4*02	82.93	C V R D G Y C T S A N C Y F F Q H W id	IgG	16
53	070521	43	V3-33*01	94.12	D2-2*03	68.00	J4*02	82.93	C V R D G Y C T S A N C Y F F Q H W id	IgG	16
54	061206	22	V1-18*01	75.56	D3-22*01	72.72	J5*02	82.35	C G R D E S S S N K S R G R R L D P W bs	IgG	17
55	061206	24	V1-18*01	84.23	D3-22*01	72.72	J5*02	88.23	C A R D E S S S N K S R R R R F D P W id	IgG	17
56	061206	36	V1-18*01	84.23	D3-22*01	72.72	J5*02	88.23	C A R D E S S S N K S R R R R F D P W id	IgG	17
57	061213	24	V1-18*01	84.23	D3-22*01	72.72	J5*02	88.23	C A R D E S S S N K S R R R R F D P W id	IgG	17
58	061213	6	V1-18*01	84.23	D3-22*01	72.72	J5*02	88.23	C A R D E S S S N K S R R R R F D P W id	IgG	17
59	061213	29	V1-18*01	84.23	D3-22*01	72.72	J5*02	88.23	C A R D E S S S N K S R R R R F D P W id	IgG	17
60	061213	18	V1-18*01	n.d.	D3-22*01	68.00	J5*02	88.23	C A R D E S S S N K S R R R R F D P W id	IgG	17
61	061206	7	V4-39*01	91.29	D1-26*01	72.72	J6*02	91.93	C A R H S C Y S A G C R Y Y Y G M D V W	IgG	18
62	061221	24	V4-4*02	91.48	D2-15*01	76.92	J3*02	90.00	C V R F I G Y C N G G S C P Y G F D I W	IgG	18
63	070510	23	V4-59*01	87.41	D3-3*01	76.92	J3*02	92.00	C A R A R V Y D F W G G R Y D A F D I W	IgG	18
64	061213	42	V5-51*01	90.32	D1-26*01	57.89	J1*01	90.38	C A R V E G T M R T G I F Y G A Y F L H W	IgG	19
65	070116	12	V5-51*01	68.57	D3-22*01	70.96	J3*02	94.00	C A R R Y Y Y D D D G Y S F R A A F D I W	IgG	19
66	070116	19	V4-59*01	94.07	D3-10*01	78.94	J6*02	79.03	C A R D R Y Y G S G S F V W R G G M D V W	IgG	19
67	070116	36	V4-39*01	90.62	D5-5*01	94.11	J4*02	91.66	C A R H E S V G D P V R G Y S N G Y P F D Y W	IgG	21

68	061206	21	V1-2*02	91.11	D5-5*01	76.00	J6*02	82.25	C A R N T Y Y F G S G S V S N Y S Y C A M D V W id	IgG	22
69	061206	26	V1-2*02	91.11	D5-5*01	76.00	J6*02	82.25	C A R N T Y Y F G S G S V S N Y S Y C A M D V W id	IgG	22
70	061213	3	V1-2*02	91.11	D5-5*01	76.00	J6*02	82.25	C A R N T Y Y F G S G S V S N Y S Y C A M D V W id	IgG	22
71	061213	8	V1-2*02	91.11	D5-5*01	76.00	J6*02	82.25	C A R N T Y Y F G S G S V S N Y S Y C A M D V W id	IgG	22
72	061213	14	V1-2*02	91.11	D5-5*01	76.00	J6*02	82.25	C A R N T Y Y F G S G S V S N Y S Y C A M D V W id	IgG	22
73	061213	16	V1-2*02	91.11	D5-5*01	76.00	J6*02	82.25	C A R N T Y Y F G S G S V S N Y S Y C A M D V W id	IgG	22
74	061213	13	V1-2*02	91.11	D5-5*01	76.00	J6*02	82.25	C A R N T Y Y F G S G S V S N Y S Y C A M D V W id	IgG	22
75	070510	6	V1-2*02	91.11	D3-10*01	76.00	J6*02	82.25	C A R N T Y Y F G S G S V S N Y S Y C A M D V W id	IgG	22
76	070510	10	V1-2*02	91.11	D3-10*01	76.00	J6*02	82.25	C A R N T Y Y F G S G S V S N Y S Y C A M D V W id	IgG	22
77	070521	22	V1-2*02	91.67	D5-5*01	76.00	J6*02	85.71	C A R N T Y Y F G S G S V S D Y F Y Y A M D V W di	IgG	22
78	070521	23	V1-2*02	91.67	D5-5*01	76.00	J6*02	85.71	C A R N T Y Y F G S G S V S D Y F Y Y A M D V W di	IgG	22
79	061213	10	V5-51*01	87.59	D2-2*01	n.d.	J6*02	85.48	C A R H Q V A P S G H A P S G V Y Y Y H G M D V W	IgG	23
80	070510	26	V1-69*06	92.80	D3-16*02	60.00	J6*02	80.64	C A R G L N Y L D T N G S P A D S Y Q Y T L D V W	IgG	23
81	070116	2	V4-61*02	92.55	D3-3*01	78.57	J6*02	91.03	C A S Q T G T L K V S Y D F W G G D Q Q N Y H Y Y A M D V W	IgG	28

No	Date of LM	Cell	V name	Identity	D name	Identity	J name	Identity	CDR3 sequence heavy chain	Isotype	CDR3 length
IBM-2 (373/96)											
1	070402	15	V3-30*18	89.13	D3-10*02	100.00	J4*02	85.41	C A K E E V A F D Y W	IgG	9
2	070402	12	V1-18*01	89.63	D3-16*02	75.00	J4*02	87.50	C A R V G R T I F D Y W	IgG	10
3	070326	17	V3-33*03	91.11	D1-1*01	n.d.	J6*03	68.52	C A K K G L Q R A L D V W	IgG	11
4	070326	13	V4-59*01	89.26	D7-27*01	71.43	J3*01	88.00	C A R A G G I Y A F D V W id	IgG	11
5	070326	26	V4-59*01	89.26	D7-27*01	71.43	J3*01	88.00	C A R A G G I Y A F D V W id	IgG	11
6	070326	19	V1-2*02	86.23	D1-7*01	66.66	J3*01	84.00	C A G A R S R L N G F D V W	IgG	12
7	070402	19	V1-2*02	92.11	D7-27*01	75.00	J5*02	80.39	C A L G P R G R G G P D Q W	IgG	12
8	070402	20	V1-2*02	86.96	D2-2*01	57.14	J3*02	82.00	C A G A K S R L N G F E I W	IgG	12
9	070326	9	V3-9*01	95.24	D6-13*01	60.00	J4*02	87.50	C A K D G T S T S W Y L D Q W id	IgG	13
10	070326	16	V3-9*01	95.24	D6-13*01	60.00	J4*02	87.50	C A K D G T S T S W Y L D Q W id	IgG	13
11	070402	17	V3-9*01	95.24	D6-13*01	60.00	J4*02	87.50	C A K D G T S T S W Y L D Q W id	IgG	13
12	070402	10	V3-9*01	95.24	D6-13*01	60.00	J4*02	87.50	C A K D G T S T S W Y L D Q W id	IgG	13
13	070402	26	V3-9*01	95.24	D6-13*01	60.00	J4*02	87.50	C A K D G T S T S W Y L D Q W id	IgG	13
14	070326	10	V1-3*01	92.63	D6-19*01	80.00	J4*02	81.25	C A R E V D S S G W F F V Y W	IgM	13
15	070326	8	V5-51*01	97.83	D6-6*01	100.00	J5*01	86.27	C A R E Y S S S S A W F D S W	IgM	13
16	070402	1	V3-11*01	87.81	D3-16*02	68.75	J4*02	83.33	C G R E R D R Y T A G T F N Y W	IgG	14
17	070402	9	V3-30*03	91.67	D6-19*01	52.38	J1*01	78.84	C A K D R S A V A G G P S D F P R W	IgG	16
18	070326	12	V4-34*01	90.37	D3-22*01	56.25	J6*02	85.48	C A R G R A G S G W S Y Y Y Y Y V M D V W	IgG	18
19	070402	6	V3-23*04	93.70	D3-3*01	71.42	J4*01	85.41	C A K D D G H D F W S G H G L L V D Y W	IgG	18
20	070326	7	V1-69*01	90.91	D2-15*01	73.33	J5*02	90.19	C A R E V V A T G I P P P G W G W F D R W	IgG	19
21	070402	23	V3-49*03	93.73	D3-3*01	91.66	J6*04	77.77	C T R D Y Y V L R F L E W V P I G M D V W	IgG	19
22	070326	22	V4-b*01	89.96	D3-22*01	84.00	J4*02	89.58	C V R D S T H N S Y D S S G Y R N L D Y W id	IgM	19
23	070402	24	V4-b*01	89.96	D3-22*01	84.00	J4*02	83.33	C V R D S T H N S Y D S S G Y R N L D Y W id	IgM	19
24	070402	3	V3-23*01	92.31	D4-23*01	70.00	J4*02	91.66	C A N R G V P H C V R L D C S G Y F D Y W	IgG	19
25	070326	21	V5-a*01	92.59	D6-6*01	94.44	J6*02	82.25	C A R L G I A A R P G V G P L Y N G M D V W	IgG	20
26	070326	14	V1-69*06	86.23	D2-21*02	71.42	J3*02	88.00	C A R G G H I A L V T A T S L P R D P F D I W	IgG	21
27	070402	5	V3-15*01	88.64	D6-13*01	52.38	J6*04	96.82	C A T F L A S A A G T D Y Y Y Y Y Y G M D L W	IgG	21
28	070326	23	V1-69*01	88.89	D3-10*01	71.42	J6*02	83.87	C A S C P P R K Y Y F G S G T R E R Y H N Y Y G M D V W	IgG	26

No	Date of LM	Cell	V name	Identity (%)	D name	Identity (%)	J name	Identity (%)	CDR3 sequence heavy chain	Isotype	CDR3 length
IBM-3 (379/96)											
1	070118	5	V3-15*01	93,97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
2	070118	14	V3-15*01	93,97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
3	070118	21	V3-15*01	93,97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
4	070118	1	V3-15*01	93,97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
5	070118	5	V3-15*01	93,97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
6	070118	10	V3-15*01	93,97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
7	070118	11	V3-15*01	93,97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
8	070118	12	V3-15*01	93,97	D4-23*01	69.23	J1*01	77.08	C N V V G R D G G S S S P H W id	IgM	13
9	070118	14	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
10	070118	17	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
11	070118	18	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
12	070118	20	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
13	070118	21	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
14	070118	22	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
15	070118	23	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
16	070118	24	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
17	070118	25	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
18	070118	26	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
19	070118	28	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
20	070118	29	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
21	070118	31	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
22	070118	30	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
23	070118	32	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
24	070207	1	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
25	070207	7	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
26	070207	8	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
27	070207	10	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
28	070207	11	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
29	070207	12	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
31	070207	19	V3-15*01	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
32	070207	20	V3-15*02	93.97	D4-23*01	69.23	J4*02	77.08	C N V V G R D G G S S S P H W id	IgM	13
30	070207	18	V3-74*02	95.45	D2-15*01	84.21	J4*02	87.50	C A R G V C S G G T C L G Y W id	IgM	13

33	070207	5	V3-74*02	95.45	D2-15*01	84.21	J4*02	87.50	C A R G V C S G G T C L G Y W id	IgG	13
34	070118	13	V3-21*01	91.85	D3-22*01	67.74	J5*02	90.20	C A K A A P Y D S S G Y Y L Y N Y F D P W	IgG	19
35	070207	32	V3-9*01	93.00	D2-15*01	71.42	J5*02	94.11	C V K E P R D C R D G D C Y R N W F D P W	IgG	19
36	070118	6	V1-69*01	88.62	D6-13*01	63.16	J6*02	82.26	C A R E A P E G D C T T I S C Y R Y N G M D V W id	IgG	22
37	070118	27	V1-69*01	88.62	D6-13*01	63.16	J6*02	82.26	C A R E A P E G D C T T I S C Y R Y N G M D V W id	IgG	22
38	070118	28	V1-69*01	88.62	D6-13*01	63.16	J6*02	82.26	C A R E A P E G D C T T I S C Y R Y N G M D V W id	IgG	22
39	070207	34	V1-69*01	88.62	D6-13*01	63.16	J6*02	82.26	C A R E A P E G D C T T I S C Y R Y N G M D V W id	IgG	22

No	Date of LM	Cell	V name	Identity (%)	D name	Identity (%)	J name	Identity (%)	CDR3 sequence heavy chain		Isotype	CDR3 length
IBM-4 (323/06)												
1	070625	17	V1-46*01	90.58	D3-9*01	75.00	J5*02	76.47	C A G A L L D N G V W A W		IgM	11
2	070704	7	V4-61*01	87.02	D4-17*01	72.73	J4*02	75.00	C A R S T W V A T Y I F W		IgG	11
3	070625	8	V4-61*01	87.33	D5-12*01	66.66	J5*02	96.07	C A R G L N L L N W F D P W		IgG	12
4	070625	10	V4-31*03	95.04	D6-19*01	86.66	J4*02	85.41	C A R R N G W Y L P D D Y W		IgG	12
5	070704	8	V5-51*01	92.03	D5-5*01	64.29	J4*02	85.42	C V R R D T N L A S G D H W		IgG	12
6	070704	14	V3-73*01	91.49	D6-6*01	66.67	J4*02	89.58	C T R R A W D G S S P F D Y W		IgG	13
7	070808	12	V1-69*01	90.00	D6-13*01	69.23	J4*02	85.42	C A R Q Q A L S P T G L E S W		IgG	13
8	070808	23	V3-9*01	94.20	D4-17*01	n.d.	J5*02	82.35	C A K A R N S D E G M L E D W		IgG	13
9	070625	27	V1-18*01	89.24	D2-15*01	72.72	J4*02	87.50	C A T T D C T G G S C Y G D R W		IgG	14
10	070704	3	V1-3*01	90.74	D6-25*01	80.00	J4*02	91.67	C A R Q G L M R R Q S S F D Y W		IgG	14
11	070704	12	V1-2*04	89.01	D6-13*01	72.22	J4*02	87.50	C A R G G S R W Y T L N W D Y W		IgG	14
12	070625	4	V4-59*01	88.41	D4-23*01	66.66	J5*02	92.15	C A R G G G F R W T N S G F D P W		IgG	15
13	070625	19	V4-34*12	91.45	D6-19*01	57.14	J4*02	81.25	C A R L C C R S A C P G G V D Y W		IgG	15
14	070625	23	V2-5*10	83.52	D6-19*01	71.42	J3*02	94.00	C A R F T A V A G T L E A F D I W		IgG	15
15	070625	30	V3-23*04	90.00	D5-12*01	63.63	J5*02	88.23	C A K G F G S G F R G G W F D P W		IgG	15
16	070625	3	V3-33*01	95.97	D1-26*01	61.53	J3*02	98.00	C A R R E L G S F Y A N D A F D I W		IgG	16
17	070625	12	V1-69*11	64.14	D2-21*01	62.50	J6*02	69.35	C A R V I G P K P V V A I G M D V W		IgG	16
18	070625	32	V3-33*01	94.44	D3-22*01	56.25	J4*02	87.50	C A R D V G H F W Q V G L G L D Y W		IgG	16
19	070625	1	V3-64*02	93.12	D6-13*01	57.14	J4*02	97.91	C A R V A S G A A G T W N Y F D Y W	id	IgM	16
20	070625	33	V3-64*02	93.12	D6-13*01	57.14	J4*02	75.00	C A R V A S G A A G T W N Y F D Y W	id	IgM	16
21	070704	2	V1-18*01	92.22	D6-6*01	77.78	J4*02	89.58	C A R V W L A G R P A P R K I D Y W		IgG	16
22	070808	28	V4-59*03	91.21	D6-6*01	66.67	J4*02	93.75	C A R M K Y S S T S G G Y S F D S W		IgG	16
23	070808	11	V1-18*01	91.11	D4-17*01	75.00	J4*02	87.50	C A R G R G G P Y G D Y A V F D F W		IgG	16
24	070704	15	V4-39*01	89.96	D3-10*01	55.17	J4*02	89.58	C A R A T P M L R G V V S L Y F V D W		IgG	17
25	070704	18	V3-23*01	90.74	D1-20*01	76.47	J3*01	92.00	C A K D L N K W N D L D H D T F D V W		IgG	17
26	070808	5	V3-9*01	91.29	D2-2*01	52.00	J4*02	87.50	C A K D N V P H P G A S R S R F D S W		IgG	17
27	070808	22	V1-18*01	90.84	D3-22*01	64.00	J4*02	91.67	C A R D L P Y Y Y D K S G R V A L D Y W	di	IgG	18
28	070625	14	V1-18*01	79.07	D3-16*01	59.09	J4*02	87.50	C A R D L P Y N Y D N V G I V S L D S W	di	IgG	18
29	070808	13	V1-69*01	91.23	D5-24*01	n.d.	J4*02	95.83	C A R A P G G F L E R G G A Y Y F D F W		IgG	18
30	070704	6	V3-23*04	89.82	D3-3*01	82.76	J4*02	97.92	C A K K G R I T S F G V V I P D Y F D H W		IgG	19
31	070704	11	V1-46*01	66.27	D7-27*01	63.64	J6*02	74.19	C A T N S Q A W D V P T V T S W T M A V W	di	IgG	19
32	070808	2	V1-46*01	84.59	D7-27*01	63.64	J6*02	74.19	C A T N S Q A W D V P T V T S W T M A V W	id	IgG	19

33	070808	4	V1-46*01	73.76	D7-27*01	63.64	J6*02	74.19	C A T N S Q A W D V P T V T X W T M A V W id	IgG	19
34	070808	30	V3-21*01	68.97	D5-24*01	n.d.	J6*02	88.71	C A R D Q I F P R S G G H F Y Y G M D V W	IgG	19
35	070625	18	V3-9*01	94.33	D6-13*01	66.66	J3*01	94.00	C A K D I E E L G I A A T F L F T A F D V W	IgG	20
38	070808	14	V3-33*01	94.62	D3-10*02	n.d.	J6*02	85.16	C A R D R F V G D A P E H F Y Y Y G M D V W	IgG	20
36	070704	34	V1-18*01	89.19	D3-16*02	n.d.	J4*02	100.00	C A R A L R D D D I R G T Y H E Y Y F D Y W	IgG	20
37	070704	20	V1-18*01	91.40	D1-26*01	60.00	J4*01	91.67	C T R I R V G V G R L G G V V G F Y Y F E Y W	IgG	21
39	070808	10	V1-69*06	94.81	D6-19*01	76.19	J6*02	91.94	C A K S A S I P V P G K M Y F Y Y Y G M D V W	IgG	21
40	070625	31	V3-23*04	94.79	D3-16*01	53.84	J6*02	87.09	C A K D E N I M T V V G G G G S L H Y Y G L D V W	IgG	23
41	070808	17	V1-24*01	90.91	D2-21*02	n.d.	J6*02	83.87	C A R V R V R V T R G L T G R T S L Y Y G L D V W	IgG	23
42	070808	7	V4-61*01	89.14	D5-5*01	70.00	J2*01	88.68	C A R S R E R I Q V W S R S P R S S G G Y F D L W	IgG	23
43	070704	5	V3-33*01	91.85	D3-22*01	67.74	J4*02	95.83	C A R D S S T M N H Y G S S V Y Y G S G S N S L D Y W	IgG	25
44	070704	29	V3-9*01	n.d.	D4-17*01	n.d.	J5*02	90.20	C A K D I F W R S A Q R N H G D Y I D G E S A F D R W	IgG	25
45	070704	22	V4-61*01	86.32	D2-21*02	62.50	J4*02	93.75	C A R E N E V A T A A L A W G A P P A A P K F Y F D S W	IgG	26

No	Date of LM	Cell	V name	Identity (%)	D name	Identity (%)	J name	Identity (%)	CDR3 sequence heavy chain	Isotype	CDR3 length
IBM-5 (243/06)											
1	070815	16	V3-74*02	94.79	D5-24*01	71.43	J4*02	83.33	C Q S S I H L G G W	IgG	8
2	070711	8	V4-59*01	92.98	D5-24*01	100.00	J5*02	86.27	C T R V S T H F D P W	IgG	9
3	070815	26	V3-23*04	87.32	D7-27*01	72.73	J4*02	85.42	C T T L S R G P G T D Y W	IgG	11
4	070711	4	V1-46*03	90.26	D6-25*01	76.92	J4*02	95.83	C V R E G S G Y K Y F D Y W	IgG	12
5	070711	20	V3-30-3*01	89.86	D4-23*01	75.00	J1*01	69.23	C A R S T V V L G A T R H W	IgG	12
6	070711	27	V1-46*03	93.41	D6-19*01	76.92	J4*02	85.42	C A R D S S D F E V F A Y W	IgG	12
7	070815	22	V3-9*01	85.66	D7-27*01	100.00	J6*02	91.94	C A K S R G D Y Y Y G L D V W	IgG	13
8	070815	6	V2-26*01	90.04	D7-27*01	72.73	J4*02	91.67	C A R I R P Q L G P D V F D F W	IgG	14
9	070815	13	V3-7*01	87.96	D3-3*01	54.17	J4*02	87.50	C V P Q L S F E W F K D L A S W	IgG	14
10	070711	32	V1-8*01	95.93	D2-15*01	71.43	J3*02	92,00	C A R D C T Y D S C R E A F D I W	IgM	15
11	070717	20	V3-30*03	96.01	D6-25*01	91.94	J6*02	62.50	C A R P R A P D Y Y Y Y G M D V W	IgG	15
12	070717	30	V4-39*01	90.11	D1-1*01	92.16	J5*02	76.92	C A R R T P T T S S R P W F D P W	IgG	15
13	070815	2	V3-23*04	74.40	D1-14*01	60.00	J4*02	93.75	C A R A S T S F S V R H F F D S W	IgG	15
14	070815	10	V3-23*01	74.40	D3-3*02	52.36	J4*02	93.75	C A R A S T S F S V R H F F D S W	IgG	15
15	070711	26	V3-33*01	89.86	D2-2*01	80.00	J4*02	81.25	C S R S R Y C S N T T C P P G D V W	IgG	16
16	070717	9	V3-7*01	92.22	D6-13*01	89.58	J4*02	61.90	C T S R P S D V S W Y L G V F D Y W	IgG	16
17	070815	3	V4-61*02	86.55	D1-26*01	58.82	J4*02	79.17	C A R A P R S P R G N F L P Y D F W	IgG	16
18	070815	8	V3-23*01	93.12	D3-10*02	66.67	J3*02	86.00	C A K D P S G G T V T G G R A F E I W	IgG	17
19	070711	1	V4-39*06	93.84	D2-2*01	64.29	J5*02	92.16	C A R D V L A V V P D G I K D W F D P W	IgG	18
20	070815	5	V1-18*01	92.14	D3-10*01	56.25	J6*02	85.48	C A R D H A R F G D L L Y Q S G M D V W	IgG	18
21	070815	28	V1-18*01	93.75	D4-23*01	92.31	J3*01	74.00	C V R D G A N S A P Q Y F Y Y G M D V W	IgM	18
22	070815	23	V3-21*01	87.64	D2-8*01	63.16	J6*03	91.94	C A R D L S Q W T G D Q D Y Y Y Y M D V W	IgG	19
23	070711	13	V4-61*02	91.40	D3-22*01	70.97	J4*02	93.75	C A R V Q G S F D G S G Y S S V D Y F D S W	IgG	20
24	070717	32	V3-30*18	92.86	D5-24*01	82.26	J6*02	82.26	C A K G E G D P I Y S W L G Y F Y G M D V W id	IgG	20
25	070717	23	V3-30*18	92.86	D5-24*01	82.26	J6*02	82.26	C A K G E G D P I Y S W L G Y F Y G M D V W id	IgG	20
26	070717	26	V1-2*02	92.67	D2-2*01	92.16	J5*02	64.52	C A R A E G S C S S V S C A R F N W F D P W	IgG	20
27	070717	6	V4-61*02	90.03	D2-21*01	93.55	J6*03	60,00	C A R A G G D G D Q L L T Y S Y Y Y M D V W	IgG	20
28	070815	14	V3-23*01	87.92	D5-5*01	90.00	J6*02	88.71	C A K A R G Y S Y G F D D D Y Y Y A M D V W	IgG	20
29	070717	11	V4-59*02	89.89	D4-23*01	88.71	J6*03	57.89	C A R G R S N R M G S H F G N N Y Y Y M D V W	IgG	21
30	070717	12	V4-61*02	89.74	D3-22*01	82.26	J6*02	77.27	C A R A G D F H D T S G F C P S C Y G L D V W	IgG	21
31	070815	4	V4-59*01	91.58	D3-3*01	80.00	J6*03	90.32	C A R D R I D T I F G V V S H S Y Y Y M D V W	IgG	21
32	070815	11	V4-61*02	71.13	D2-2*01	62.96	J3*02	94.00	C A K T X R D G D S D P V P V P I E A F D I W	IgG	21

33	070815	15	V4-61*02	90.97	D2-2*01	62.96	J3*02	94.00	C A K T A R D G D S D P V P V P I E A F D I W	IgG	21

No	Date of LM	Cell	V name	Identity	D name	Identity	J name	Identity	CDR3 sequence heavy chain	Isotype	CDR3 length
PM-1 (350/95)											
1	070226	3	V3-7*01	90.22	D6-13*01	100.00	J4*02	79.16	C T S G H Y G C W	IgG	7
2	070219	18	V3-73*01	90.22	D1-26*01	66.66	J4*01	75.00	C T K M G F T T E N F W id	IgG	10
3	070219	34	V3-73*01	90.22	D1-26*01	66.66	J4*01	75.00	C T K M G F T T E N F W id	IgG	10
4	070214	17	V1-69*06	89.26	D2-21*01	n.d.	J6*02	81.03	C S R D R Y S D G M D V W id	IgG	11
5	070214	18	V1-69*06	89.26	D2-21*01	n.d.	J6*02	81.03	C S R D R Y S D G M D V W id	IgG	11
6	070219	32	V5-51*01	89.49	D1-1*01	72.72	J4*02	91.66	C V R R P T R G Q F D S W	IgG	11
7	070226	23	V1-24*01	97.83	D3-16*01	n.d.	J6*02	79.03	C V R G L R E Y V M D V W	IgG	11
8	070219	40	V4-31*01	89.71	D2-15*01	72.72	J2*01	83.01	C A R G F W K V G S F D L W	IgG	12
9	070307	5	V4-59*01	87.50	D4-11*01	100.00	J5*01	82.35	C A R D L M T T D R G F D L W	IgG	13
10	070110	3	V5-51*01	92.59	D2-15*01	55.55	J5*02	94.11	C A R H S S L D L S S W F D P W id	IgG	14
11	070110	4	V5-51*01	92.59	D2-15*01	55.55	J5*03	94.11	C A R H S S L D L S S W F D P W id	IgG	14
12	070214	13	V1-69*01	90.53	D6-13*01	69.23	J3*01	88.00	C A H Q T G Y S L S A T F D V W	IgG	14
13	070307	8	V3-9*01	95.70	D3-10*01	83.33	J6*02	87.09	C V K S G Y F S Y F Y S M D V W	IgG	14
14	070214	25	V1-46*01	88.15	D3-16*01	62.50	J6*02	90.32	C V R G G A Q T Y Y Y H G M D V W	IgG	15
15	070214	28	V3-23*01	92.31	D4-17*01	66.66	J2*01	96.22	C A K R R G D Y F P D W Y F D L W	IgG	15
16	070219	37	V3-9*01	97.44	D3-22*01	80.00	J6*02	83.87	C A K D S C G G G S C Y G M D V W	IgG	15
17	070226	15	V4-61*08	94.93	D4-4*01	75.00	J5*02	100.00	C A R A H T T T T T D N W F D P W	IgG	15
18	070214	16	V1-18*01	91.57	D6-19*01	62.50	J5*02	96.07	C G R M L V G G A W F G N W F D P W	IgG	16
19	070214	19	V4-31*06	88.61	D3-16*01	60.00	J6*02	83.87	C A R V S P P R G T Y Y Y S M D V W	IgG	16
20	070214	33	V4-34*01	92.88	D4-23*01	63.15	J5*02	92.15	C A R G R L F G G T F H N W F D P W	IgG	16
21	070226	9	V5-51*01	72.34	D3-22*01	70.00	J6*02	80.64	C A R Q M R V G G T P N H G M D V W id	IgG	16
22	070226	12	V5-51*01	94.87	D3-22*01	70.00	J6*02	80.64	C A R Q M R V G G T P N H G M D V W id	IgG	16
23	070214	4	V1-69*04	86.96	D2-15*01	76.00	J4*02	87.50	C S R S P F E G Q C F G G R C W Y L D S W	IgG	19
24	070307	12	V3-30-3*01	98.17	D5-24*01	66.66	J6*02	95.16	C A T P T P D E M G Y F Y Y Y H G M D V W	IgM	19
25	070214	31	V3-7*01	95.93	D6-13*01	68.42	J4*02	97.91	C A R G R S S W T P W A T R T D Y F D F W	IgG	19
26	070226	17	V1-69*01	88.15	D2-15*01	75.00	J6*02	83.87	C A R G P G Y F N G D R F F Y N G L D V W	IgG	19
27	070214	9	V3-9*01	88.15	D2-2*03	70.00	J5*02	92.15	C A K D F G S S V A I V A G S I H Y F D P W	IgG	20
28	070214	26	V5-51*01	87.68	D3-10*01	77.41	J4*02	81.25	C V R H G A W Y G S E S N Y N V G L L D Y W	IgG	20

29	070214	6	V4-59*02	88.52	D3-22*01	74.19	J4*02	93.75	C A R L R R A P H Y D S T G Y Y H T Y F F D Y W di	IgG	22
30	070307	9	V4-59*02	89.74	D3-22*01	77.41	J4*02	97.91	C A R L R R A P H Y D S T G Y Y H T Y F F D Y W di	IgG	22
31	070110	8	V3-9*01	92.98	D3-3*01	89.47	J6*02	85.48	C A R D K G R E F Y D F W S G H S Y H F G M D V W	IgG	23
32	070214	22	V3-30*03	89.26	D2-21*01	58.82	J6*02	85.48	C A R D G G G P S H D S V V L R H Y F Y G L D V W	IgG	23

No	Date of LM	Cell	V name	Identity (%)	D name	Identity (%)	J name	Identity (%)	CDR3 sequence heavy chain	Isotype	CDR3 length
PM-2 (249/06)											
1	070725	12	V4-30-2*01	88.44	D2-21*02	77.78	J4*02	91.67	C V R R R D S Y Y D Y W	IgG	10
2	070719	7	V3-30*04	86.36	D7-27*01	100.00	J3*02	76.03	C A K G D A A G A M D N W	IgG	11
3	070725	6	V4-59*01	91.49	D2-15*01	75.00	J5*02	72.55	C A R V S L G R G A L N W	IgG	11
4	070725	7	V1-2*02	88.41	D6-25*01	75.00	J4*02	87.50	C F I S G Y D S V L D F W	IgG	11
5	070719	16	V3-23*01	88.53	D4-17*01	66.67	J4*02	83.33	C A K N P D D Y S I F D F W	IgG	12
6	070719	22	V1-46*01	88.89	D6-13*01	100.00	J6*03	80.65	C A R G S S Y T H Y M D V W	IgG	12
7	070719	28	V3-33*01	96.63	D6-25*01	83.33	J4*02	100.00	C A R D R Y S S D Y F D Y W	IgG	12
8	070725	9	V1-18*01	96.88	D3-22*01	71.43	J4*02	97.92	C A R G R R L L K Y F D Y W	IgG	12
9	070725	16	V3-43*01	93.50	D3-16*02	63.64	J4*02	85.42	C A K D M G G D R L L D S W	IgG	12
10	070719	15	V3-23*01	90.04	D1-14*01	61.54	J3*02	90.00	C A R Q D I S G P T A F D M W	IgG	13
11	070725	22	V5-51*01	95.97	D6-19*01	100.00	J4*02	83.33	C A R S R R G I A V A A D Y W	IgG	13
12	070719	2	V3-23*04	95.34	D1-1*01	87.50	J4*02	100.00	C A K D S N W N D P N Y F D Y W	IgG	14
13	070719	14	V4-59*01	95.88	D3-10*01	55.00	J4*02	75.00	C A R G G A R G I F S P L T A W	IgG	14
14	070719	5	V3-23*01	96.97	D6-25*01	73.33	J4*02	87.50	C A K D P E H N S S G A T V D Y W	IgG	15
15	070719	33	V3-74*01	99.63	D3-16*02	66.67	J4*02	81.25	C A R D R E T A L G G A G V D Y W	IgM	15
16	070725	14	V3-7*02	91.48	D3-22*01	62.50	J4*02	89.58	C A R R G H S S G S Y R P F D Y W	IgG	15
17	070725	4	V1-18*01	87.50	D1-14*01	60.00	J4*02	97.92	C A T G L F T G I I S T H Y F D S W	IgG	16
18	070725	5u	V7-4-1*02	91.85	D2-15*01	82.76	J3*01	78.00	C A R G K Y C S G G N C Y G R Y W D F W id	IgG	18
19	070725	2	V7-4-1*02	91.23	D2-15*01	82.76	J3*01	78.00	C A R G K Y C S G G N C Y G R Y W D F W id	IgG	18
20	070719	32	V4-59*01	96.30	D2-2*01	70.37	J2*01	96.23	C A R A P G I L V I P P T P F W Y F D L W	IgG	19
21	070725	18	V3-48*02	92.03	D3-22*01	62.50	J1*01	92.31	C A R D A S P E D Y S T S G D I E Y F H H W	IgG	20
22	070719	27	V1-18*01	93.33	D5-24*01	54.55	J6*02	87.10	C A R D H V R V L V E V S D R Y K I G Y Y G L D V W	IgG	24

Supplementary tables: showing in detail the results of genetic analyses of the Ig gene from isolated plasma cells from IM biopsies

(id) identical sequences; (di) different sequences but clonal related; (bs) bad sequence

7 Curriculum vitae

Name:	Christoph Stiehler
Date of birth:	September 26, 1977 (Mülheim an der Ruhr)
Nationality:	German
Marital status:	married, one child

Education:

since 09.2003	**Ph.D. Thesis** (Dr. sc. nat.), University Hospital Zurich, Department of Neurology, Clincal Neuroimmunology Unit commitee members: Prof. Dr. Norbert Goebels, Prof. Dr. Adriano Aguzzi, Prof. Dr. Burkhard Becher Title: "Analysis of the plasma-cell repertoire in inflammatory myopathies" Participation at the International Ph.D. Program in Neuroscience (ZNZ)
07.2002-07.2003	**Master`s Thesis**, ALTANA Pharma AG Konstanz, Department of Biotechnology, Supervisor: Prof. Dr. Klaus P. Schäfer Title: "Cloning and expression of Lungsurfactant protein A2 by using the expression system 293 EBNA", mark: sehr gut (CH: 6.0)
10.1998-06.2002	**Studies in biology**, University of Konstanz Bachelor (10.1998-10.2000) Master studies (10.2000-07.2002) Master exam in biochemistry and immunology (03.07.2002)
08.1997-08.1998	Community service (Evangelische Akademie Mülheim an der Ruhr)
06.1997	**A-level**, Gesamtschule Saarn (Mülheim an der Ruhr)

Stipends:

- Hartmann-Müller Stiftung 06/2006-
- Zentrum für Neurowissenschaften Zürich (ZNZ) 05/2004-05/2006

Publications:

Christoph Stiehler, Hans-Christian von Büdingen, Norbert Goebels
Analysis of the plasma-cell repertoire in inflammatory myopathies

manuscript in preparation

Oral presentations:

ZNZ Symposium 2005:
Molecular and functional analysis of the B- and plasma-cell repertoire in dermatomyositis and multiple sclerosis

Laser Microdissection User Meeting 2006:
Molecular and functional analysis of the plasma-cell repertoire in myositis syndromes

Abstracts:

Joint Meeting 2007 of the
Swiss Society for Neuroscience, National Center of Competence in Research: Neural Plasticity and Repair, and Swiss Multiple Sclerosis Society:
Molecular and functional analysis of the plasma-cell repertoire in myositis syndromes

6th day of Clinical Research, University of Zurich:
Molecular and functional analysis of the plasma-cell repertoire in myositis syndromes

ZNZ Symposium 2006:
Molecular and functional analysis of the plasma-cell repertoire in dermatomyositis